Therapeutic effects of the traditional medicinal plant Ipomoea stolonifera for the treatment of liver diseases

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Therapeutic effects of the traditional medicinal plant *Ipomoea stolonifera* for the treatment of liver diseases

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Chapter 1

General introduction

Traditional Chinese medicine for treatment of liver diseases

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The liver is the largest internal organ involved in metabolism and detoxification in humans. It metabolizes and stores nutrients (protein, glycogen, vitamins, lipids, cholesterol), it is responsible for the synthesis of bile acids from cholesterol and the synthesis of many plasma proteins and it detoxifies xenobiotics, such as drugs and alcohol [1, 2]. These activities can be grouped into (a) synthetic functions, (b) metabolic functions, (c) detoxification. Not surprisingly, impairment of the normal function of the liver can lead to serious disruption of homeostasis and liver diseases are therefore characterized by high morbidity and mortality. In traditional Chinese medicine, the heart is considered the 'King' or 'Supreme Commander' over body functions, whereas the liver is considered as the 'General' or 'Long-range Planner'. These terms are paralleled in Western physiology, e.g. the liver plays a critical role in maintaining body energy ('Qi') and the balance between 'Yin' and 'Yang' [3, 4]. The 'planning' capacity of the liver can be translated to maintaining blood quality (detoxification, plasma protein synthesis), digestion and metabolism of carbohydrates (glucose), and storage of important nutrients (vitamins, lipids, glycogen). Sometimes it is difficult to translate the terminology from Chinese traditional medicine to Western physiology. In traditional Chinese medicine, the liver 'has its opening in the eyes'. In '*Neijing*', it is stated 'liver qi communicates with the eyes', suggesting that the eyes are closely linked to the liver [5]. This could be related to the fact that the eyes are nourished by liver (blood) and that the liver is the storage organ for vitamin A, a crucial vitamin in vision, e.g. vitamin A deficiency leads to (night) blindness. Furthermore, the eye is an indicator for liver function: yellow colorization of the eyes may reflect jaundice. Additionally, according to traditional Chinese medicine, the liver plays a central role in harmonizing the emotional flow and governing mental and spiritual functions [6]. Likewise, intense emotions like chronic rage, complaining, despair, jealousy often adversely affects liver function. Thus, a healthy liver is indispensable for normal body function, both from the Western and the Eastern perspective.

In humans, the liver is composed of two liver lobes and has a dual blood supply: oxygen-rich blood is supplied by the hepatic artery that accounts for 25% of the liver blood supply, whereas 75% of the blood supply comes from the gut via the portal vein [7]. The blood from the portal vein contains nutrients absorbed from the intestinal lumen, but also potentially harmful substances absorbed from the gut, e.g. toxins, pathogens, xenobiotics and gut-derived endotoxins. The liver is therefore the first organ exposed to toxic substances from the gut and therefore susceptible to damage induced by these noxious compounds. Fortunately, the liver is well-equipped to deal with these challenges: the liver is the primary site of detoxification of many toxic compounds and the clearance of pathogens and gut-derived endotoxins. Furthermore, the liver has a remarkable capacity to adapt and withstand various forms of injury through regenerative repair. The equilibrium between cell death, proliferation and differentiation is crucial for the maintenance of tissue homeostasis throughout life. Within the liver, various phenotypically distinct cell types are responsible for the maintenance of this delicate equilibrium. These cell types include parenchymal cells (mainly hepatocytes and cholangiocytes) and non-parenchymal (mesenchymal) cells, including endothelial cells, stellate cells, Kupffer cells and lymphocytes (Fig. 1).

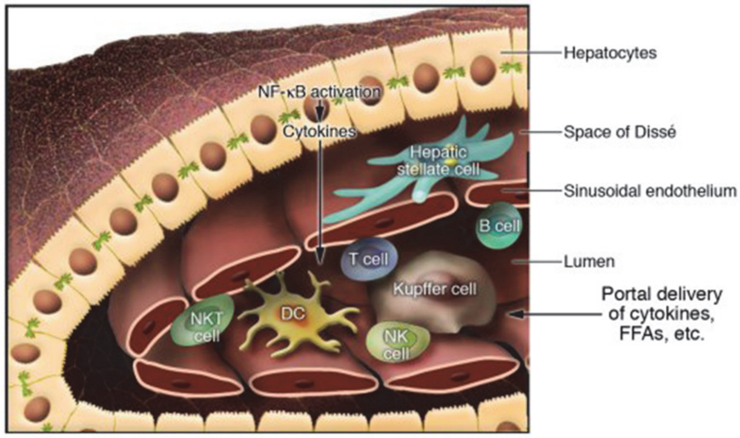

Figure 1. The location and structure of various cell types in the liver, which contains hepatocytes, liver sinusoidal endothelial cells, hepatic stellate cells, and resident hepatic macrophages (Kupffer cells), B and T cells, NK and NKT cells, hepatic dendritic cells (DCs). Some of cells participate in inflammatory and immune responses. Reprinted with permission from Steven E. Shoelson © 2006 American Society for Clinical Investigation [8] .

First, we introduce these cell types and their functions in the liver.

Parenchymal cells (hepatocytes and cholangiocytes)

Within the liver there are two types of polarized epithelial cells: hepatocytes and cholangiocytes (also known as biliary epithelial cells). In terms of mass and number, the hepatocyte is the predominant liver cell type, comprising around 70% of the total number of cells in the liver and approximately 80% of the total liver mass [2, 9, 10]. Cholangiocytes line the bile ducts and modulate bile flow [11]. Both cell types, hepatocytes and cholangiocytes, are crucial for liver function and homeostasis. Damage to one of these cell types is the cause of most liver diseases leading to inflammation and fibrosis [12]. The hepatocytes are responsible for many liver-specific functions, such as intermediary metabolism, detoxification of toxic compounds, synthesis of bile acids and many plasma proteins, and generation of bile acid-dependent bile flow. In the healthy liver, very few of the hepatocytes are progressing through the cell cycle, the vast majority remains in a quiescent state (G0) [13]. However, hepatocytes retain the ability to re-enter the cell cycle in response to a liver insult, e.g. after partial resection of the liver [14] or severe liver injury [15]. In most liver diseases, in particular inflammatory liver diseases, it are mostly the hepatocytes that perish. Loss of hepatocytes occurs by cell death. The major types of cell death are apoptosis and necrosis although alternative forms of cell death also occur, e.g. autophagy. Although apoptosis and necrosis have clear definitions, it is increasingly recognized that they represent two extremes of a continuum. Indeed, intermediate forms of cell death like necro-apoptosis (necroptosis) displaying features of both apoptosis and necrosis have been described [16]. Apoptosis, or programmed cell death, is characterized by cell shrinking, membrane blebbing, DNA condensation, nuclear fragmentation, and finally the formation of apoptotic bodies. Mitochondrial morphology remains intact and the cellular content remains confined and does not spill into the circulation [17, 18]. The morphological events are accompanied by the activation of specific proteases, called caspases, in particular the downstream effector caspases-3, -6 and -7, which cleave cellular structures and proteins resulting in cell death. In contrast, necrosis, or 'passive' cell death, is characterized by cell swelling, rupture of the plasma membrane with subsequent spilling of cellular content into the circulation, resulting in inflammation and mitochondrial swelling. Nuclear morphology remains intact [19]. Autophagy, or cellular self-digestion (Greek for "self-eating"), is cell death mediated by the lysosomal degradation of cellular components. Three subtypes of autophagy are distinguished: macroautophagy, microautophagy, and chaperone-mediated autophagy [20, 21]. At present, the exact causal role of autophagy to liver damage is not clear. For example, autophagy could act both as an agonist and as an antagonist of cell death depending on the experimental context. Although apoptosis is crucial for embryonic development and normal tissue homeostasis, including liver homeostasis, excessive apoptosis in liver diseases is detrimental. The predominant mode of hepatocyte cell death is dependent on the liver disease, e.g. necrotic cell death is the predominant mode of cell death in acute acetaminophen intoxication and many chronic liver diseases, whereas apoptotic cell death is observed in acute (viral) hepatitis and nonalcoholic steatohepatitis (NASH). Most liver diseases, however, display a mixed phenotype of cell death [22, 23].

Non-parenchymal cells

Macrophages

In the liver, macrophages can be broadly defined as either resident macrophages (Kupffer cells) or monocyte-derived macrophages [12], which are responsible for the elimination of pathogens (bacteria) and clearance of foreign compounds, e.g. apoptotic bodies by phagocytosis through a variety of receptors. Kupffer cells reside in the hepatic sinusoids, in close contact with other circulating immune cells, so they participate in local immune responses and are well-positioned to eliminate invading pathogens and remove obsolete cells (erythrocytes) or cell fragments (apoptotic bodies). Macrophages are also the driving force of inflammation in many liver diseases. Upon activation by various triggers (e.g. bacterial products) the macrophages, including the Kupffer cells, release pro-inflammatory cytokines (Tumor necrosis factor (TNF)-α and IL-1β), chemokines (CCL2 and CCL5), other inflammatory mediators (prostaglandins and leukotrienes) and reactive oxygen species (ROS). These secreted products influence not only the hepatocytes, but also stellate cells, portal myofibroblasts and other immune cells. In chronic liver diseases, the continuous inflammatory state leads to a fibrogenic response, since the products released from Kupffer cells contribute to the activation, proliferation and survival of stellate cells and myofibroblasts, in part via the activation of the transcription factor Nuclear Factor-κB (NF-κB) in HSCs and myofibroblasts [24]. On the other hand, macrophages also possess a latent capacity to revive damaged tissue by secreting matrix metalloproteinases (MMPs) that degrade newly synthesized scar matrix. The protease activity of MMPs is inhibited by concurrent production of tissue inhibitors of metalloproteinases (TIMPs) by myofibroblasts and macrophages.

Hepatic stellate cells (HSCs)

Hepatic stellate cells (HSCs) account for 5%–8% of the cells in the liver and are located in the space of Disse, the space between the sinusoidal endothelial cells and the basolateral surface of hepatocytes [25, 26]. In the healthy liver, HSC contain large quantities of retinyl-esters packed in big lipid droplets that serve as the main storage site of vitamin A in the body. In liver fibrogenesis, the key event is the transdifferentiation of these “quiescent”, vitamin A-containing HSCs into proliferative myofibroblasts that progressively lose their vitamin A content. Liver myofibroblasts are derived mainly from the activation of quiescent HSCs, but may also arise from other sources, e.g. portal fibroblasts [27, 28] and bone marrow-derived cells [29, 30]. Although the exact initial events in the activation of HSCs are still not completely elucidated, various cytokines, growth factors and reactive oxygen species (ROS) are involved. Upon activation, the HSCs lose their vitamin A content and demonstrate increased proliferation, contractility, matrix production and pro-inflammatory signaling. The accumulation and enhanced density and cross-linking of hepatic extracellular matrix (ECM) components produced by myofibroblasts leads to fibrosis and is characterized by increased matrix stiffness, hampered blood flow through the liver, portal hypertension and complications like variceal bleeding. Regression of liver fibrosis can be achieved when the disease-causing factor(s) can be eradicated. Activated HSCs either undergo apoptosis or revert to a quiescent phenotype characterized by downregulation of markers of fibrosis such as type I collagen (col1a1) and alpha-smooth muscle actin (α-SMA) [31, 32]. Subsequently, other cell types in the liver contribute to the clearance of HSCs, e.g. apoptotic HSCs are phagocytized by macrophages and senescent HSCs are removed by natural killer cells [33-35]. Therefore, removal and/or “de-activation” of HSCs/myofibroblasts is increasingly recognized as an essential step towards the resolution of liver fibrosis.

Natural Killer (NK) cells and Natural Killer T-cells (NKT cells)

The immune system plays an important role in maintaining tissue homeostasis and dysregulation of this system may contribute to several liver diseases. In the context of liver diseases, NK cells and NKT cells are important. NK and NKT cells both contain a characteristic set of markers for NK cells (mouse：DX5 or NK1.1; rat: NKR-P1A; human: CD56) , while NKT cell, in addition, express T cell markers (like

CD1) [7, 36, 37]. In acute or chronic liver injury, both cell types play an important role in the first-line innate defense against viral infection and tumor transformation, which is associated with many liver diseases [38, 39]. The distribution of NK and NKT cells is different in the livers of mice, rats and humans. NKT cells account for 30–40% of total lymphocytes in mouse liver and only 5-10% of total lymphocytes in rat and human liver [40]. Hepatic NK and NKT cells have many similar functions after activation, such as the production of pro-inflammatory cytokines (IFN-γ and IL-4) and the killing of virus-infected cells and tumor cells [7]. The major mechanism by which NK cells lead to hepatocyte cell death and liver injury is via the release of TRAIL and granzyme B, whereas NKT cells release predominantly FasL [41]. NK cells also appear to have a negative regulatory effect on fibrogenesis and have been reported to directly kill early-activated or senescent hepatic stellate cells [35, 42, 43]. This is probably mediated by IFN-γ, released by activated NK cells, that inhibits stellate cell activation and amplifies NK cell cytotoxicity against stellate cells [44-46]. On the other hand, IFN-γ is also a proinflammatory cytokine that induces hepatocyte cell-cycle arrest and apoptosis [47, 48]. Therefore, inhibition of the production of IFN-γ by NK cells and NKT cells could be beneficial for the survival of hepatocytes and allow hepatic regeneration, but could also promote fibrogenesis. Interestingly, experimental evidence suggests that IFN-γ that is specifically targeted to stellate cells suppresses liver fibrosis in mice [49].

Liver sinusoidal endothelial cells (LSEC)

Liver sinusoidal endothelial cells (LSEC) line the hepatic sinusoids. They have a unique morphological phenotype that is characterized by open (non-diaphragmed) fenestrae and a lack of basement membrane [50]. This morphology facilitates an optimal exchange of nutrients, oxygen and waste products between the sinusoidal blood and space of Disse. LSECs are also responsible for removing soluble macromolecular and colloidal waste products (smaller than 100 nm) [9, 51]. Furthermore, LSEC have the appropriate location, together with Kupffer cells, to play a role in the clearance of pathogens and viruses. Giugliano et al [52] reported the release of antiviral exosomes from the LSECs that amplify the antiviral activity of interferons in hepatocytes. Additionally, differentiated LSECs that maintain their normal fenestration and function prevent HSC activation. In cocultures of LSECs and HSCs, the presence of LSECs maintains the quiescent state of HSC [53]. Once LSEC differentiation is lost, they promote HSC activation and liver fibrosis. Therefore, differentiated LSECs have a function as gatekeeper in the fibrotic process [50].

Liver diseases

A number of different conditions (inflammatory disorders and metabolic disorders) affect liver functions, which are associated to multiple cell types discussed above. Traditionally, liver diseases are categorized into acute liver failure (ALF) and chronic liver failure (CLF) [54, 55]. The essential feature of ALF is the abrupt loss of hepatic function due to the loss of large numbers of hepatocytes [56]. This rare but sudden syndrome in a patient with no history of liver disease, but causes severe complications (i.e. jaundice, coagulopathy, hepatic encephalopathy, and even multi-organ failure) [57]. Viral hepatitis remains the cause of a high proportion of cases of ALF, which is common caused by infections of virus A, B, C and E, and leads a high rate of death world while. And virus hepatitis predominates in developing countries more than in developed countries [58]. It is not optimistic that of the 350 million hepatitis B virus (HBV) carriers worldwide, one-third reside in China [59]. That means 780 000 people die every year due to complications of hepatitis B [60], the number in China is nearly a half reported by National Health and Family Planning Commission of China. Besides virus, toxins, drugs, immunological attacks and various chemicals could also cause hepatitis, and each of these stimulations could be possible to result in CLF.

CLF, a progressive and slow deterioration, often occurs in people with incurable chronic liver disease or stable cirrhosis. CLF leads to metabolic disorders of various toxins and presents irreversible chronic loss of liver function, which usually is accompanied by continuous inflammation [61]. Alcohol consumption has been globally identified as a major risk factor in liver diseases, such as alcoholic fatty liver disease (AFLD) and ASH [62]. The characteristic of non-alcoholic fatty liver disease (NAFLD) and AFLD are fat accumulation in the liver (steatosis) and with alcohol consumption 20-30 g/day (AFLD) and without any evidence of chronic liver diseases and lower alcohol drink (NAFLD). Furthermore, advanced stages of these two liver diseases are NASH and ASH [63]. The consequence and final stage of various CLF are generally liver fibrosis and liver cirrhosis [64]. Actually, fibrosis is the liver's protective response to injury, and this intrinsic reaction will protect the liver against further damage from the structure and functions of differential cell types. So the liver can be reversed by itself from fibrosis even cirrhosis to normal architecture when the insults removed or the causes treated. The successful reversion of fibrosis and cirrhosis should include the degradation of excess ECM [64], and matrix-metalloproteinases (MMPs) associated with tissue inhibitors of metalloproteinases (TIMPs) make main contributions to degrade excess ECM. But the objective situations usually do not look good. Accumulation of deposited extracellular matrix (types I and type IV fibrillary collagens), server liver injury and uncontrolled immune responses to inflammatory, will finally cause irreversible liver fibrosis characterized by cellular activation of HSCs (see above). And therefore, this is one main topic focused in this thesis. Cirrhosis is defined as fibrous scarring of the hepatic parenchyma resulting in nodule formation and collapse of liver structures. Now it is fourth cause of morbidity and mortality in central Europe [65]. Collectively, these are among the key initiating risk factors for two types of primary liver cancer: hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) [66].

Additionally, in 1995, a third type of liver failure acute-on-chronic liver failure (ACLF) was described for the first time [67]. The definition of this new entity is based on patients with 3 key syndromes: liver cirrhosis presenting with acute decompensation, a high rate of organ failure and a high short –term of mortality [68, 69]. Unfortunately, the classification of ACLF is still not universal defined so far. However, the further improvement concerning of ALF according to its prognosis will be beneficial to clinical managements on these patients.

Signal transduction in hepatic injury

Although the liver has a high regenerative capacity, the loss of viable and functional hepatocytes may lead to liver failure, depending on the type and severity of the injury. The massive and acute loss of a large proportion of functional hepatocytes, as in acute liver failure or fulminant hepatitis, may not be compensated by hepatocyte regeneration because of an inability of the hepatocytes to regenerate in these conditions or because the loss of hepatocytes is too extensive. On the other hand, in chronic liver failure there is a continuous, but more moderate loss of hepatocytes, which can be compensated by regeneration. However, in chronic liver injury, the continuous presence of an insult may result in sustained inflammation and fibrogenesis, eventually exceeding the regenerative capacity of hepatocytes and leading to liver failure. In addition, the continuous regenerative trigger may lead to hepatocellular carcinoma (HCC). To understand the pathogenesis of liver diseases we will now first review some of the key signaling mechanisms involved in liver inflammation and liver fibrosis.

Inflammatory cytokines, interferons and chemokines

In acute and/or chronic liver injury, the cellular wound-healing response is accompanied by the production of inflammatory cytokines and chemokines. The release of these soluble factors contributes to the wound healing response and the clearance of foreign substances and cell debris and is therefore beneficial. However, excessive and/or prolonged production of these factors may be detrimental to hepatocytes and also lead to uncontrolled activation and proliferation of stellate cells. In fact, some of the released cytokines, such as TNF-α, interleukin (IL)-1, and IL-6 play a role in the proliferative response of hepatocytes and induce the production of so-called acute phase proteins that are important in maintenance of homeostasis. Other cytokines, like FasL, TNF-α and other ligands of the so-called "death receptor family", can induce apoptotic cell death in hepatocytes. Hence, TNF-α plays a crucial role in the inflammatory response of the liver, since it has both proliferative and cell protective effects by activating the transcription factor NF-κB and it has pro-apoptotic properties (when cells are unable to activate NF-kB) by activating death receptor pathways. Furthermore, TNF-α controls the production of many other cytokines. The biological effects of TNF-α are mediated by its receptors TNF receptor 1 (TNF-R1) and TNF-R2. Signaling by TNF-R1 and TNF-R2 is achieved by recruitment of adaptor proteins that bind to the cytosolic domains of the receptors (like MyD88) and initiate signal transduction events [70]. IL-1β is another potent inflammatory cytokine that signals through the IL-1 receptor 1 (IL-1R1), leading to an inflammatory cascade. Increased levels of IL-1β contribute to the progression of several chronic inflammatory liver diseases, including NASH and Alcoholic steatohepatitis (ASH).

Interferons (IFNs, IFN-α/β and IFN-γ) comprise a family of proteins, which interact with cells via distinct cellular receptors. Type II IFN (IFN-γ) is produced mainly by macrophages, NK cells, and T lymphocytes [71], while type I IFN is produced by fibroblasts, epithelial cells and hepatocytes [72]. IFNs have been tested as anti-fibrotic agents in patients with moderate fibrosis. It was demonstrated that IFN-γ displays anti-fibrotic effects and suppresses proliferation and α-SMA expression in HSCs. Furthermore, IFN-γ activates NK cells that in turn induce death of HSCs [73]. Additionally, IFN-β can inhibit the repeated hepatocellular injury and reduce liver fibrosis induced by Con A [74]

Chemokines are small proteins (8–13 kDa) and are categorized into 4 different families: CC, CXC, CX3C, C) [75]. It has been shown that chemokines and their receptors play a seminal role in the pathogenesis of various acute and chronic liver diseases. Chemokines, by virtue of their capacity to attract inflammatory and immune cells to sites of inflammation, not only drive inflammation and immune responses, but also participate in fibrogenesis and cancer [76]. CCL2 (monocyte chemotactic protein-1, MCP-1) is one of the best characterized chemokines. In the chronically inflamed liver, CCL2 is secreted by many different cell types [77, 78], but mainly by Kupffer cells and HSCs, which contribute to the recruitment and accumulation of macrophages and monocytes into the liver. During development of non-alcoholic fatty liver disease (NAFLD) and NASH, CCL2 and its receptor are upregulated in the liver, where it promotes hepatic and systemic inflammation related to metabolic disorders [79-81].

Transcription factor Nuclear Factor- κB (NF-κB)

NF-κB is a ubiquitous transcription factor that is activated by a variety of stimuli, including cytokines (TNF-α, Transforming growth factor (TGF)-β) and reactive oxygen species (ROS). NF-κB is a key regulator of many cellular responses involved in inflammation and stress in chronic liver diseases (e.g. viral hepatitis, liver fibrosis and NAFLD) [82]. The 'classical' NF-κB is a heterodimer of the subunits p50 (NF-κB -1) and p65 (Rel-A). Additional subunits exist giving rise to alternative homo- and heterodimers. Normally, NF-κB is retained in an inactive form in the cytoplasm through association with one of the IκB inhibitory proteins, including IKK-α or IKK-β, and this interaction blocks the ability of NF-κB to translocate to nucleus. Upon stimulation of appropriate receptors, e.g. binding of TNF-α to its receptor, the IκB kinase (IKK) complex is phosphorylated and activated. The IKK complex is composed of a regulatory subunit (IKK-γ) and two kinase subunits (IKK-α, IKK-β) that are responsible for the phosphorylation of IκB. Phosphorylation of IκB leads to its poly-ubiquitination and subsequent proteolytic degradation. These events allow the translocation of NF-κB from the cytoplasm to the nucleus where NF-κB acts by inducing the transcription of target genes [83]. The target genes of NF-κB include inflammation-related genes, like cytokines, including TNF-α itself and chemokines, but also anti-apoptotic genes. Therefore, NF-κB has a pivotal role in the inflammatory response. Therefore, there is a tremendous, ongoing effort to identify and develop drugs that target NF-κB activity. However, since NF-κB is associated with pro-survival activity, the continuous activation of this transcription factor in inflammatory disorders might also predispose for an increased risk for hepatocellular carcinoma.

c-Jun N-terminal kinase (JNK)

C-Jun N-terminal kinases (JNKs), members of the mitogen-activated protein kinase (MAPK) family, are involved in multiple signaling cascades that lead to hepatocellular cell death. It is a pro-apoptotic kinase and is involved in hepatocellular injury in inflammatory, metabolic and fibrotic liver diseases. The liver expresses two JNK isoforms, JNK1 and JNK2, but not JNK3 [84]. Cross-talk exists between JNK and TGF-β and platelet-derived growth factor PDGF-mediated Smad 2/3 signaling [85]. In addition, the interplay between NF-κB and JNK has been extensively investigated and reviewed [70, 86, 87]. Importantly, it has been demonstrated that NF-κB generates its survival signals in part via inhibition of the prolonged activation of JNK by TNF-α [70], and in turn, activation of JNK is prolonged in NF-kB-deficient cells [88].

Toll-Like Receptors (TLRs)

Toll-Like Receptors (TLRs) are essential receptors in the host defense against pathogens in the early innate immune response. TLRs have the ability to recognize highly conserved structural motifs known as pathogen-associated microbial patterns (PAMPs), which include various bacterial cell wall components, such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipopeptides. LPS, the TLR4 ligand, induces inflammatory signals in multiple cell types in the liver. The activation of TLR4 on Kupffer cells leads to the production of many proinflammatory cytokines, including TNF-α, IL-1β, CCL2 and CCL20 [89-91]. In addition, TLR4 signaling also modulates TGF-β-mediated HSC activation in liver fibrosis [92, 93]. Several therapeutic agents targeting the TLRs are now under pre-clinical and clinical evaluation. However, given the complexity of signaling and the multitude of TLRs, interventions in these pathways may act as double-edged swords either promoting or inhibiting disease progression.

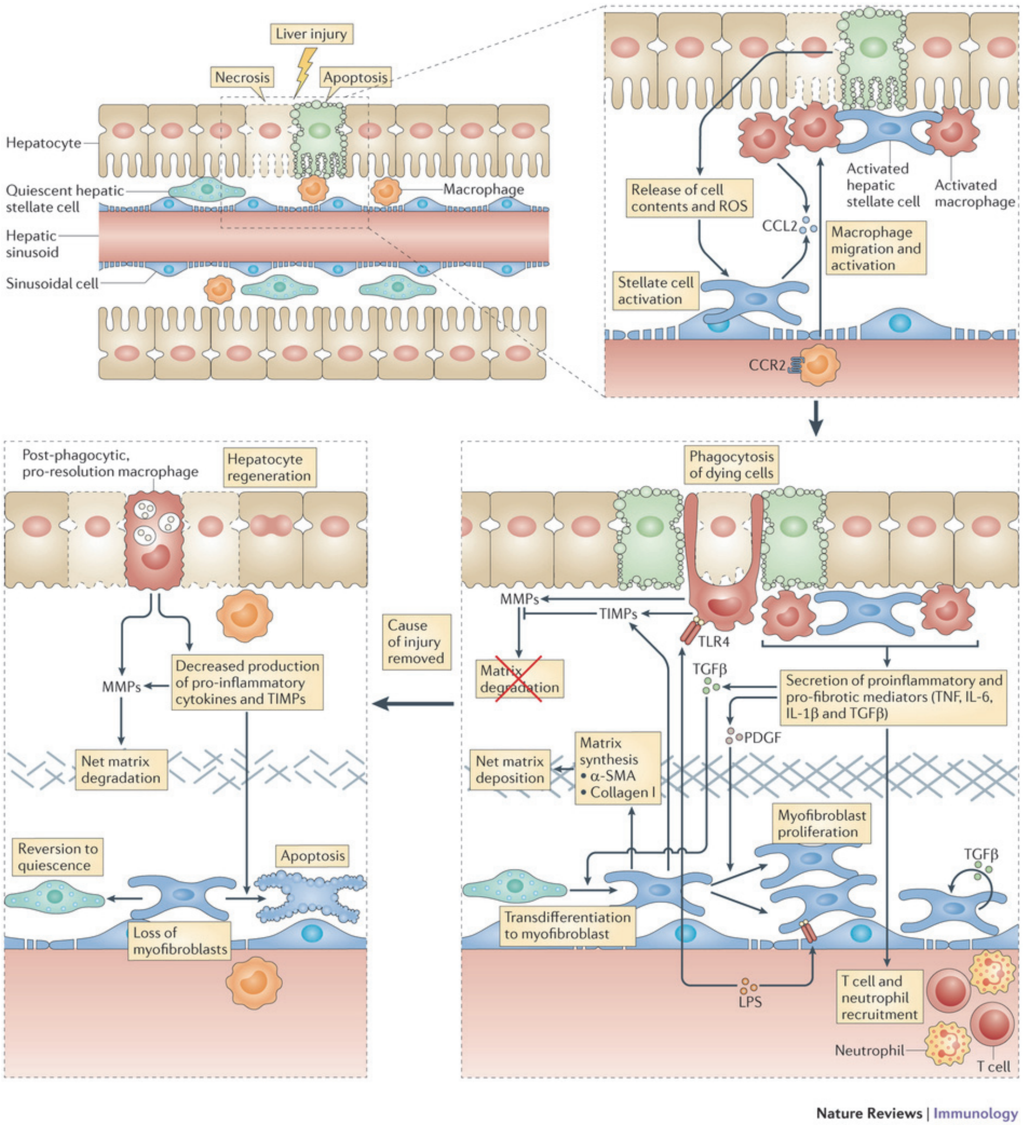

Figure 2. Cascade of signals following liver injury CCL2 and CCR2: CC-chemokine ligand 2; (CCL2)–CC-chemokine receptor 2. IL, interleukin; TNF, tumor necrosis factor. Reprinted with permission from Antonella Pellicoro © 2014 Nature Publishing Group [12].

Liver injury leads to necrotic and/or apoptotic cell death of parenchymal cells. Phagocytosed cell debris leads to activation of macrophages and HSCs, which is amplified by the release of cytokines and reactive oxygen species. These pro-inflammatory mediators also recruit T cells, neutrophils and lymphocytes, amplifying the inflammatory response. Increased gut permeability, e.g. during acute inflammation, leads to increased translocation of gut-derived endotoxins, via the portal blood, to the liver. In the liver, endotoxins and other bacterial products bind to CD14 and TLR4 receptors on Kupffer cells, triggering an inflammatory response, including the activation of stress-activated and mitogen-activated protein kinase (p38, JNK), and NF-κB [94]. Among the secreted pro-inflammatory and pro-fibrotic mediators is TGF-β, one of the key factors driving fibrogenesis. TGF-β is mainly produced by inflammatory and immune cells and directly promotes liver fibrosis via stimulation of HSC transdifferentiation into myofibroblasts, a process characterized by increased expression of α-SMA and collagen type I. The growth factor PDGF also stimulates proliferation of myofibroblasts. Reversal of matrix deposition and scar accumulation is regulated by MMPs and its natural inhibitors (TIMPs). Upon removal or termination of the underlying triggers for liver injury (alcohol, fat, hepatitis viruses), the liver is, to some extent, capable to regenerate and resolve fibrosis [95-97].

Therapeutic drugs for liver diseases

Liver diseases are difficult to treat by medication. Yet, several drugs are now prescribed for various liver diseases: for NASH and NAFLD, statins, such as pravastatin and atorvastatin, are the most prescribed drugs. They act by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and thereby reduce cholesterol synthesis. These drugs improve liver histology and serum markers in patients and are in general safe, with only rare examples of toxic side-effects [98]. In recent years, the anti-diabetic drug metformin is increasingly prescribed for NASH/NAFLD patients with good results [99]. In the treatment of HCC, sorafenib is commonly used for unresectable/non-ablatable or advanced-stage HCC. It is an inhibitor of various tumor growth-promoting tyrosine kinases (raf-, VEGF receptor, and PDGF receptor) [100]. Ursodeoxycholic acid is used in the treatment of cholestatic diseases [101]. Lamivudine, a deoxycytidine analogue that has a high oral bioavailability is active against hepatitis B virus (HBV) [102]. The major mechanism of lamivudine is blocking viral DNA synthesis and inhibiting HBV replication. Finally, hepatitis C virus infection is treated by a cocktail of drugs that inhibit various HCV proteins, including the HCV polymerase and protease. Unfortunately, many of the currently available therapies are not very effective (with the exception of the novel treatment for HCV [103]). Furthermore, most of these therapeutic interventions target the agents that cause the disease, but not target the already ongoing inflammation, cell death and/or fibrogenesis. In fact, there are currently no drugs available that directly target liver fibrosis, while more general anti-fibrotic drugs have a significant risk for side effects [104], for instance pirfenidone that may lead to gastrointestinal syndromes[15] (inhibiting TGF-β expression and activation) [105, 106], and Imatinib (targeting the non-receptor tyrosine kinase cAbl) [107]. For end stage liver failure, the only effective therapeutic option is liver transplantation. Since the supply of donor organs is inadequate to meet the growing demand, this is also not an optimal solution for many patients. Therefore, complementary and alternative medicines have gained increasing attention for the treatment of liver diseases.

Herbal products for the treatment of liver disease

An important group of complementary and alternative medicines are herbal products. For thousands of years, herbal products have been used by indigenous people worldwide to treat a plethora of illnesses. In the past few decades, these natural products also caught attention in Western countries. Many cultures have used traditional medicine for the treatment of diseases, e.g. in China (traditional Chinese medicine, TCM), Japan (Kampō medicine), Korea (traditional Korean medicine), Indonesia (Jamu), India (Ayurvedic medicine), North America (phytotherapy), and Europe (herbalism) [108]. The medical application of herbal products has fostered research into the chemical and biological analyses of numerous prescriptions [109-112]. Simultaneously, it has initiated a successful approach to novel drug identification and development through the isolation and purification of active ingredients from herbal products [113] [114]. A few examples of herbal products that have been used in the treatment of liver diseases will now be discussed.

Silymarin (Milk thistle)

Silymarin, the extract of the Milk thistle (*Silybum marianum*) contains four flavonoids: isosilybin, silybin, silychristin and silydianin (Fig. 2). The major component silybinin (also called silybin) accounts for 90% of the herb's flavonoid content in most preparations. Silymarin is found commonly throughout Europe, Asia and North America. Its extracts were already used as early as the 4th century B.C. and became a single-herb remedy for liver disease and jaundice in the 1960s [115, 116]. One of the reasons why silymarin became so popular is because of its postulated mechanisms of actions that include antioxidant activity, anti-inflammatory activities and hepatoprotective actions with little or no toxicity [117-119]. Studies in cell culture and animal models demonstrated that silymarin enhances the activity of ribosomal RNA polymerase in hepatocytes and decreases hepatic injury by inhibiting glutathione depletion in hepatocytes and inhibiting the production of leukotrienes, prostaglandins and TNF-α by Kupffer cells [120]. Furthermore, sylimarin has been shown to block proliferation of HSCs [121] and to inhibit TGF-β1-induced collagen secretion [122]. Several clinical trials have shown the beneficial effects of silymarin in liver diseases, including liver cirrhosis, non-alcoholic fatty liver disease, hepatitis B/C virus infection and liver cancer [123, 124]. The beneficial effects of silymarin on parameters of liver injury (AST, ALT) are thought to be caused by modulation of immune responses and improvement of anti-oxidant defenses by increasing superoxide dismutase (SOD) activity and increasing glutathione levels. Although silymarin shows low intestinal absorption after oral administration, dosages of 420 mg/day are well-tolerated and have shown significant therapeutic effects in the treatment of liver cirrhosis and viral hepatitis. Low doses of silybin (20 to 48 mg/kg/day) are used as an antidote for acute *Amanita phalloides* (deathcap mushroom) poisoning [118], which occurs frequently in Europe, especially in Germany. The main mechanism of this protective action is repressing the uptake of amatoxin (the toxin of *Amanita phalloides*) via competitive inhibition of the transporter OATP1B3 and thereby decreasing the concentration of amatoxin in the enterohepatic circulation [125]. Although the value of silymarin in the treatment of chronic liver failure is still unclear because of the lack of well-controlled clinical trials, it remains the best currently available therapy for *A. phalloides* poisoning.

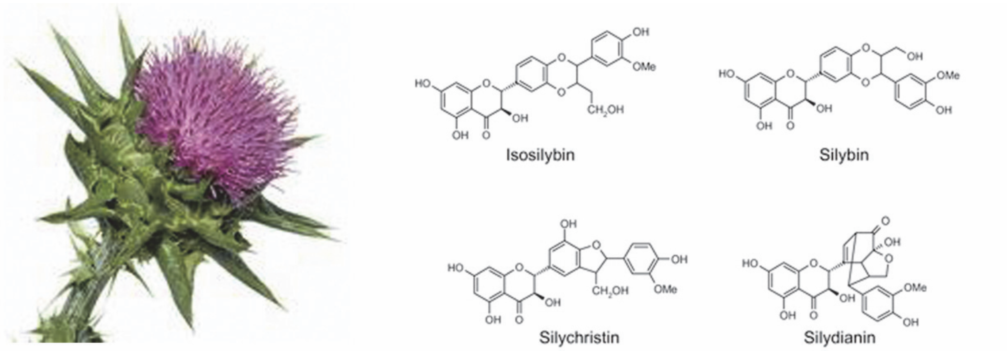

Figure 3. The milk thistle (*Silybum marianum*) and the chemical structures of its four major flavonoid constituents. Taken from: http://www.besmartstayhealthy.com/milk-thistle.html

Glycyrrhizin (licorice root extract)

Glycyrrhiza glabra (licorice root), is a perennial herb that is widely cultivated in Southeast Europe and Western Asia (Fig. 3). It has been used for centuries in traditional medicine to treat cough, bronchitis, gastritis and liver inflammation [126, 127]. It is available as over-the-counter medication in liquid, powder and pill forms in the United States. Glycyrrhizin is the aqueous extract of licorice root. It is the conjugate of two molecules of glucuronic acid and one molecule of 18β-glycyrrhetic acid (GA) [128]. Other components of the aqueous extract are several flavonoids, isoflavonoids, hydroxy-coumarins and β-sitosterols [129]. Glycyrrhizin prevents or attenuates liver injury in several animal models of liver disease, as well as in in liver disease patients in clinical trials. Glycyrrhizin targets the hepatocyte membrane [130, 131] and this property is being exploited by developing hepatocyte-specific delivery systems incorporating glycyrrhizin [132, 133]. In Japan, Neominophagen C, a more potent formulation that contains glycyrrhizin, cysteine and glycine, is administered parenterally to treat acute and chronic hepatitis [134, 135]. The use of glycyrrhizin in acute and chronic hepatitis is based on its hepatoprotective, immunomodulatory and anti-inflammatory effects. In animal models, glycyrrhizin has been demonstrated to suppress the inflammatory response via PI3K-mediated inhibition of NF-κB activation, resulting in diminished production of inflammatory cytokines like TNF-α, IL-4, IL-6 and IL-1β and inflammatory mediators like nitric oxide, reactive oxygen species and prostaglandin E2 [136]. Glycyrrhizin inhibits infiltration of inflammatory cells into the liver and reduced liver injury in the concanavalin A (ConA) model [137]. In the model of D(+)-galactosamine (GalN)/lipopolysaccharide (LPS)-induced fulminant hepatitis, glycyrrhizin prevented High-Mobility Group Box 1 (HMGB1)-dependent hepatocyte apoptosis [138] and glycyrrhizin also reduced ischemia/reperfusion (I/R)-induced liver injury [139]. Glycyrrhizin also inhibits ConA-induced proliferation of splenic CD4(+)T and enhances IFN-γ and IL-10 expression in these cells via JNK, ERK and PI3K/Akt-dependent mechanisms. Moreover, glycyrrhizin inhibited ConA-induced phosphorylation of JNK, ERK and PI3K/Akt in this study, suggesting that glycyrrhizin attenuates liver injury and fibrosis via modulation of the CD4(+)T cell response [140]. Of note, some studies demonstrated that several nuclear receptors, e.g. glucocorticoid receptor, pregnane X receptor (PXR), estrogen receptor and peroxisome proliferator-activated receptor gamma (PPAR-γ), are regulated by glycyrrhizin, which may also be pharmaceutical targets of glycyrrhizin. Moreover, glycyrrhizin was shown to lower AST and ALT levels in a long-term clinical study of HCV patients in Japan and the Netherlands [145, 146]. In addition, glycyrrhizin exerts anti-inflammatory effects on SAH-induced vasospasm and attenuates the expression of PPARs, especially PPAR-γ, which corresponds to the severity of SAH-related inflammation.

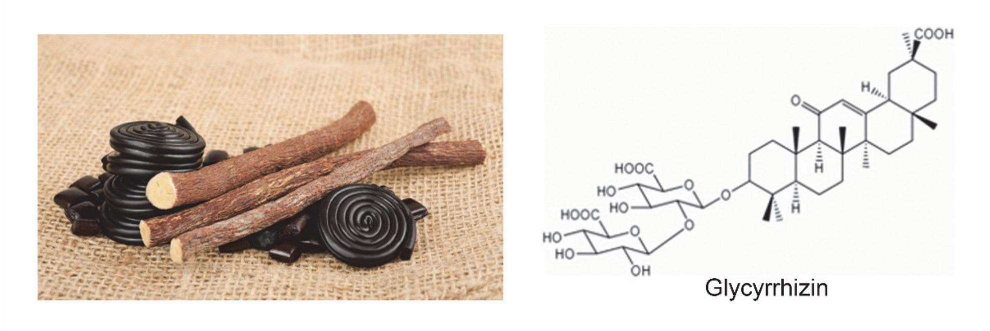

Figure 4. Glycyrrhiza glabra and its main aqueous extract component: glycyrrhizin. Taken from: https://www.organicfacts.net/health-benefits/herbs-and-spices/health-benefits-of-licorice.html and http://drugdiscoveryopinion.com/2009/03/liquorice-does-allsorts/

Traditional Chinese Medicine

Traditional Chinese medicine (TCM) is an ancient, holistic treatment system established through empirical evaluation. It aims to restore energy (Qi) and balance (Yin and Yang) through the use of complex medicinal plants, fungi, animal products and minerals. TCM typically contains many components and therefore appears quite different from the therapeutic approaches of Western medicine. TCM is a holistic approach, using individualized herbal remedies to target complex syndromes and to help the body regain balance and harmony [147]. Thus, in TCM, the entire human body is treated as a single unit and the selected herbs are able to act in a complementary fashion. Supporting evidence for TCM was documented already in 16 B.C. in the book *Pen Tsao*, describing more than 300 herbs for medical treatment. The most important basic theory of TCM is the *Jun-Chen-Zuo-Shi* principle of combining different medicinal compounds in a specific manner when preparing TCM formulations (*Fufang*). *Fufang* means that each component in a TCM formulation has its own biological target and the combined action of all components on different biological targets is the principle of *Fufang*. For instance, most herbal mixtures comprise 4 to 5 herbs of which 1 or 2 are pharmacologically active compounds present in high doses. The herb that targets the major symptom of the disease is called *Jun* (“Emperor” or “King”). The remaining components, *Chen* (“Minister”), *Zuo* (“Assistant”) and *Shi* (“Courier”), have supporting functions, such as strengthening *Jun*’s therapeutic effects, eliminating possible adverse or toxic effects of the *Jun* and/or *Chen* components or working synergistically with *Jun* [148].

It has been demonstrated that at least 42% of patients with liver diseases use some form of traditional medicine, 20% of whom use herbal preparations [134]. The following section of the introduction describes some therapeutic examples of complex herbal medicines that are currently being investigated in clinical trials.

Xiao-Chai-Hu-Tang (TJ-9)

Xiao-Chai-Hu-Tang (TJ-9, Sho-saiko-to in Japanese), a classic herbal composite formula, is widely used in China and in Japan to treat liver diseases. It consists of 7 herbal components: bupleurum root, pinellia tuber, scutellaria root, jujube fruit, ginger rhizome, ginseng root and glycyrrhiza root. The six purified components of TJ-9 are baicalein, baicalin, Saikosaponins, ginsenosides, wogonin, and gingerol (Fig. 4). It is administered orally in doses of up to 7.5 g to patients with chronic viral liver diseases and the concentration of each active component is regulated [149]. In 1995, Oka *et al* [150] performed a prospective, randomized, non-blind controlled study in 260 patients, which demonstrated a significant beneficial preventive effect of TJ-9 on HCC development, in particular in patients that were HBs-Ag negative [119]. In addition, an anti-fibrotic effect of TJ-9 in two rat models of liver fibrosis (diethylnitrosamine- and pig serum-induced liver fibrosis) was demonstrated [151, 152]. More recently, Takahashi *et al*. reported their findings of TJ-9 treatment in non-alcoholic steatohepatitis (NASH) [153]. TJ-9 significantly alleviated necroinflammation and fibrosis in the liver in this mouse model of NASH. The beneficial effects of TJ-9 were (partly) caused by increased expression of peroxisome proliferator-activated receptor-gamma (PPARγ) and reduced expression of TNF-α and IL-6. Among the components of TJ-9, saikosaponin-C and saikosaponin-D, the isomers of saikosaponin, induced apoptosis of lymphocytes, partly by increasing levels of c-myc and p53 mRNA and decreasing levels of Bcl-2 mRNA [154]. Thus, TJ-9 may hold great promise for the prevention and/or treatment of complex liver diseases.

Figure 5. The seven herbal components of Kampo TJ 9 include bupleurum root (at the bottom), jujube fruit (left bottom), pinellia tuber (bottom right), ginger rhizome (on the top), glycyrrhiza root (up left), scutellaria root (top right), and ginseng root (on the left). Taken form: http://kampo.ca/herbs-formulas/formulas/shosaikoto/

Fuzheng Huayu (FZHY)

Similar to TJ-9, Fuzheng Huayu (FZHY) is a complex preparation of Chinese herbal medicine consisting of six other medicinal herbs (Fig. 6): Radix Salvia Miltiorrhizae, Cordyceps, Semen Persicae, Gynostemma Pentaphyllammak, Pollen Pini and Fructus Schisandrae Chinensis [155]. It is approved by the State Food and Drug Administration (SFDA) of China as an antifibrotic medicine [156] and it has also been reported to promote blood flow and reduce markers of liver injury and cirrhosis (Child-Pugh score) [157]. In clinical trials, FZHY was shown to significantly improve clinical symptoms and liver function, to reverse hepatic fibrosis and to decrease portal pressure in patients with chronic hepatitis B and liver cirrhosis [158]. In animal models of liver fibrosis, FZHY decreased α-SMA expression, attenuated ECM deposition and inhibited the TGF-β1 signal transduction pathway [159, 160]. FZHY also induced apoptosis in rat HSC-T6 cells by activating p38 and inhibiting SAPK/JNK [161, 162], which is opposite to its effect on hepatocytes. In addition, FZHY has been shown to have antifibrotic properties by increasing the production of IFN-γ by hepatic NK cells [163] and to have anti-oxidant and anti-inflammatory properties by reducing the expression of cytochrome P450 2E1 and TNF-R1 [164]. FZHY has successfully completed phase 2 clinical trials in the United States [147, 165].

Figure 6. The product information of Fuzheng Huayu capsules of the Institute of Liver Diseases, Shanghai University of Traditional Chinese Medicine. The constituents of FZHY, clockwise from the top, are Cordyceps sinensis mycelium (tonifying spirit), Semen Persicae (Zuo: 'assistant', supports the function of Salvia), Fructus Schisandrae Chinensis (prevents liver injury), Gynostemma Pentaphyllammak (removing heat and toxicity), Pollen Pini (nourishes the liver) and Radix Salvia Miltiorrhizae (prevents blood stasis).

Ipomoea stolonifera

Ipomoea pes caprae

Ipomoea Scarlett OiHara

Ipomoea tricolor

Figure 7. Members of the *Convolvulaceae* family. *Ipomoea*, a large genus of more than 500 species, full of rich colors, grows in the tropical and subtropical zones throughout the world. Most of these species can adapt to harsh environments, such as salty and dry coastal areas. Taken from http://toptropicals.com/

Ipomoea stolonifera (beach morning-glory)

Ipomoea stolonifera (IS) is a coastal herb belonging to the *Convolvulaceae* (morning glory) family (Fig. 6). Like other herbal products, the exact composition of this herb can vary with location, season and even the altitude where the herbs grow [166, 167]. Even within one genus, extracts of flowers with different colors may vary in their medicinal properties [168, 169]. *I. stolonifera* normally occurs in tropical and sub-tropical regions, along sandy dunes and beaches, e.g. the Chaoshan area in Guangdong [170]. Traditionally, it has been used for the treatment of sunstroke, colitis, dysentery and fish puncture wounds, but *IS* has also been used for the treatment of inflammatory disorders, like rheumatoid arthritis [171]. The actual effective constituents of *I. stolonifera* are beginning to be elucidated, which is part of the work described in the thesis. In previous research, the n-butanol extract of *I. stolonifera* (BE-IS) was prepared and characterized [171]. This extract showed strong anti-inflammatory activity in the carrageenan-induced paw edema test in rats. In order to identify novel and active compounds, the BE-IS was subjected to chromatographic analysis and five major constituents were identified based on nuclear magnetic resonance (NMR) spectrum and mass spectrometry (MS) analysis: the coumarins scopoletin, esculetin and umbelliferone and the flavonoids hesperetin and curcumin. Multiple activities for these compounds have been described, including antioxidant and anti-inflammatory properties, but the therapeutic effectiveness of BE-IS and its five purified components in liver injury has not been elucidated yet.

Summary

Traditional medicine holds great promise: the multicomponent nature of traditional medicines has great advantages in the therapy of many diseases. However, there are also challenges: reconstitution of complex mixtures from synthetic, purified compounds may not always reflect the original natural components and minor components may be 'missed'. On the other hand, processing of original preparation to obtain extracts that can be administered easily (e.g. preparing extracts), may change the composition of the original preparation or alter the chemical structure of the active ingredients. In addition, there may be batch-to-batch variation between different preparations depending on e.g. season or geographical location. A major challenge will be to identify the optimal composition of complex mixtures. It is often not clear which are the most potent active ingredients or which combination has major therapeutic effects. Therefore the complexity and reproducible preparations of natural products still provides significant scientific challenges [172]. The strict regulation and standardization of all aspects of medicinal plant preparation by the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) hampers the registration and manufacture of herbal products.

Another challenge is bridging the gap between experimental studies and clinical application. Although the past few decades have witnessed an enormous increase in knowledge from experimental studies, this has not been translated in an increase in clinical studies. The reasons for this 'translational gap' are mentioned above: the complexity of natural products and the difficulty in manufacturing specified and reproducible preparations of natural products. Nevertheless, the prospects for successfully treating patients with liver diseases with validated preparations of natural products have never been brighter.

Scope of the thesis

The aim of this thesis is to investigate the butanol-extract (BE-IS) form *Ipomoea stolonifera* and five purified compounds of this extract on acute and chronic liver diseases, in particular inflammatory liver diseases and liver fibrosis *in vivo* and *in vitro.*

In **Chapter 1**, we provide an overview of the most important liver cell types and signaling cascades involved in inflammatory liver diseases and an overview of traditional medicine. In **Chapter 2**, we evaluated the hepatoprotective effect of BE-IS and its five individual components on the inflammatory response and bile acid-induced cell death in primary hepatocytes *in vitro*. We demonstrate that each component of BE-IS displayed differential properties. Following this initial screen, hesperetin was selected for detailed investigation. In **Chapter 3**, we evaluated the therapeutic properties of hesperetin in two mouse models of fulminant hepatitis: the concanavalin A (Con A) model and the D-galactosamine /lipopolysaccharide (D-GalN/LPS) model. We demonstrate a profound therapeutic effect of hesperetin in these models of fulminant hepatitis. Following promising effects of the BE-IS component esculetin on hepatic myofibroblasts *in vitro*, in **Chapter 4** we evaluated esculetin for anti-fibrotic effects in a mouse model of liver fibrosis (CCl_4-induced hepatotoxicity). In **Chapter 5**, a follow-up study of the anti-fibrotic effect of esculetin is presented, in which the optimal route of administration and duration of treatment for esculetin were determined. In **Chapter 6**, the results of this thesis are discussed focusing on strategies for the treatment of inflammatory liver disorders and hepatic fibrosis and providing an outlook for the application of our findings in the treatment of human liver diseases.

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Chapter 2

Raw extract from the Chinese herb *Ipomoea stolonifera* and its purified components have anti-inflammatory and cytoprotective effects on rat hepatocytes

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Abstract

Background: *Ipomoea stolonifera* (IS) is a Chinese herb that has potent anti-inflammatory properties in traditional medicine. Liver diseases are almost invariably accompanied by inflammation and loss of liver function due hepatocyte cell death. Here, we analyzed the effect of the n-butanol extract from IS (BE-IS) and five compounds purified from BE-IS (scopoletin, esculetin, umbelliferone, hesperetin and curcumin) on the inflammatory response and bile acid-induced cell death in hepatocytes and macrophages.

Methods: Primary rat hepatocytes were isolated from Wistar rats and treated with BE-IS and its 5 purified constituents to analyze the effects on cytokine mixture (CM)-induced inflammation and glycochenodeoxycholic acid (GCDCA: 50 μmol/L)-induced cell death. The mouse macrorophage cell line RAW264.7 was treated with lipopolysaccharide to induce inflammation. iNOS and HO-1 mRNA expression were used as markers for inflammation and oxidative stress, respectively. Apoptosis was quantified by caspase-3 activity assay and determination of poly (ADP-ribose) polymerase cleavage and necrosis by lactate dehydrogenase (LDH) release.

Results: BE-IS and its purified compounds all inhibited CM-induced inflammation to variable extents. CM-induced iNOS mRNA expression was significantly reduced by curcumin, hesperetin and BE-IS. HO-1 mRNA expression was increased by BE-IS, curcumin and hesperetin. BE-IS dose-dependently repressed GCDCA-induced apoptosis, independent of p38, ERK or PI3k signaling. BE-IS and its constituents do not induce necrotic cell death.

Conclusion: Raw extracts of *Ipomoea stolonifera* and the purified compounds scopoletin, umbelliferone, hesperetin and curcumin have anti-inflammatory and cytoprotective effects on rat hepatocytes and macrophages. BE-IS is therefore a potential source for therapeutics to treat chronic inflammatory liver diseases.

Introduction

The liver plays a fundamental role in many vital functions such as nutrient metabolism, detoxification, energy supply, vitamin A storage and plasma protein synthesis. Maintenance of these liver functions is therefore crucial to maintain homeostasis and health. In many liver diseases, hepatocytes, the functional parenchymal liver cells, are continuously exposed to potentially toxic factors such as cytokines as a result of inflammation, reactive oxygen species (ROS) as a result of inflammation and/or metabolism of xenobiotics and bile acids as a result of inflammation and/or cholestasis [1, 2]. This exposure to toxic compounds may result in increased apoptotic and/or necrotic cell death of hepatocytes and loss of functional liver mass, compromising the important liver-specific functions and homeostasis [3-5]. The continuous loss of functional hepatocytes, accompanied by inflammation, may evolve in liver fibrosis, cirrhosis and eventually primary hepatocellular carcinoma. Alcohol abuse, obesity, viral hepatitis, drug intoxication and genetic disorders are the most important causes of chronic liver diseases [6, 7]. Currently, effective therapy, aimed at preventing or attenuating inflammation and loss of functional liver cells is lacking. Thus, there is an urgent need to develop novel and more effective therapies to improve hepatocyte survival and to decrease inflammation in acute and chronic liver diseases.

Ipomoea stolonifera is a medicinal herb from the Chaoshan area, Guangdong province, China. It has been widely used as a traditional medicine to treat inflammatory disorders, especially rheumatoid arthritis [8, 9]. In previous research, we have investigated the n-butanol extract from *Ipomoea stolonifera* (BE-IS) in mice. It was observed that BE-IS possessed potent anti-inflammatory activity in acute and chronic inflammation models [10]. Through classical chemical separation techniques, five active compounds have been purified from BE-IS: members of the class of coumarins, Scopoletin, Esculetin and Umbelliferone and members of the class of flavonoids, Hesperetin and Curcumin (Fig.1). All these compounds possess anti-inflammatory effects both *in vivo* and *in vitro* (unpublished data). Although these compounds exhibit several bioactive properties [11-14], only a few studies have been conducted to evaluate its hepatoprotective effects in liver diseases.

In the present study, we aim to evaluate the effect of BE-IS and its 5 purified components on primary hepatocytes, and to test their potential to protect hepatocytes against cytokine mixture (CM)-induced inflammation and bile acid-induced cell death. In addition, we tested the anti-inflammatory effects of BE-IS and its components in the murine macrophage-derived cell line (RAW264.7). BE-IS and its individual compounds showed a cytoprotective effect on hepatocytes via inhibition of caspase-3 activation and an anti-inflammatory effect by repressing iNOS mRNA expression in both hepatocytes and RAW264.7 macrophages. BE-IS could also prevent LDH release from hepatocytes. Hence, this class of natural products demonstrate important cell protective and anti-inflammatory actions in liver diseases that justify its evaluation in *in vivo* models and, possibly, in clinical trials.

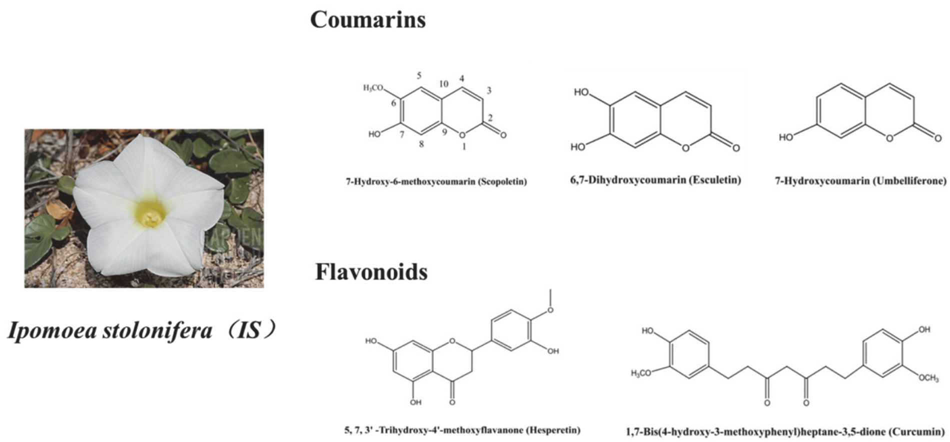

Figure 1. Structures of the five purified compounds from the n-butanol extract from *Ipomoea stolonifera* (BE-IS).

Materials and Methods

Cell isolation and culture

Primary hepatocytes were isolated from male Wistar rats (220-250g) by a two-step collagenase perfusion procedure as described previously [15]. Experiments were performed following the guidelines of the local Committee for care and use of laboratory animals of the University of Groningen. Hepatocyte viability was more than 85% as determined by trypan blue staining. 112500 cells per cm^2 were plated on Vitrogen® (Cohesion Technologies Inc, Palo Alto, CA, USA) coated plates in William's E medium (Life Technologies Ltd; Breda, The Netherlands) supplemented with 50mg/mL gentamycin (Life Technologies Ltd) and penicillin-streptomycin-fungizone (Lonza, Verviers, Belgium). During the attachment period (4 hrs) 50 nmol/L dexamethasone (Sigma, St Louis, USA) and 5% fetal calf serum (Hyclone/Thermo) were added to the medium.

The mouse macrophage cell line RAW264.7 was obtained from ATCC (Manassas, VA, USA) and cultured in DMEM Glutamax medium (Life Technologies Ltd; Breda, The Netherlands) supplemented with 10% v/v heat-inactivated fetal calf serum and 1% gentamycin. Both hepatocytes and RAW264.7 cells were cultured at 37^0C in a humidified atmosphere containing 5% CO_2.

Experimental design

All experiments were started at least 4 hours after the attachment period. Cultured primary hepatocytes were first pretreated with the five purified compounds and BE-IS for 30 mins, and then challenged by a cytokine mixture (CM: 20 ng/ml murine

TNFα, 10 ng/ml human IL-1β and 10 ng/ml rat IFN-γ) for 6 hrs or glycochenodeoxycholic acid (GCDCA: 50 μmol/L) for 4 hrs. Lipopolysaccharide (LPS Escherichia coli 055:B5, Sigma 1 μg/ml) was used to induce an inflammatory response in RAW264.7 macrophages. BE-IS and its individual components were added 30 min prior to the addition of LPS to RAW264.7 cells. Signal transduction pathways were blocked using ERK1/2 inhibitor (U0126 at 10 mmol/L) (Promega, Madison, USA), p38 inhibitor (SB203580 at 10mmol/L) (Biomol, Plymouth Meeting, USA) and PI3K inhibitor (LY 294002 at 50 mmol/L, Calbiochem). All inhibitors were added 30 min before adding stimuli. H_2O_2 at 5 mmol/L was used as a positive control for necrotic cell death. Every experimental condition was performed in triplicate wells and each experiment was repeated at least four times using hepatocytes from different isolations. BE-IS was prepared as described previously [10].

Western blot analysis

20 microgram of total cell lysate protein was used for SDS-PAGE followed by semi dry-blotting to transfer protein to nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA). Following blocking, membranes were probed with primary antibodies: rabbit anti-poly (ADP-ribose) polymerase (PARP) polyclonal antibody (1:1000, Cell Signaling Technology, Beverly, Massachusetts, USA) and mouse anti-GAPDH (1:10,000, Calbiochem, VWR, the Netherlands CB1001), Protein bands were detected using a Chemidoc XRS system (Bio-Rad). Protein band intensities were quantified by Image Lab (Bio-Rad).

RNA isolation and quantitative real time PCR

RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed on 2.5 μg of total RNA using random nanomers (Life technologies) in a final volume of 50 μL. Real time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems) using the Taqman protocol. This protocol includes an initiation phase of 10 min at 95°C, followed by 40 cycles (15 seconds at 95°C, and 1 minute at 60°C). 18S mRNA levels were used as an internal control. Relative gene expression was calculated using the ΔΔCt method. The primers (Invitrogen) and probes (Eurogentec) used are: sense iNOS: 5′-GTG CTA ATG CGG AAG GTC ATG-3′; antisense iNOS: 5′-CGA CTT TCC TGT CTC AGT AGC AAA-3′; probe iNOS: 5′-CCC GCG TCA GAG CCA CAG TCC T-3′. Sense HO-1: 5′-CAC AGG GTG ACA GAA GAG GCT AA-3′; antisense HO-1: 5′-CTG GTC TTT GTG TTC CTC TGT CAG-3′; probe HO-1: 5′-CAG CTC CTC AAA CAG CTC AAT GTT GAG C-3′. Sense 18S: 5′-CGG CTA CCA CAT CCA AGG A-3′; anti-sense 5′-CCA ATT ACA GGG CCT CGA AA-3′; probe 18S: 5′-CGC GCA AAT TAC CCA CTC CCG A-3′.

Apoptosis and necrosis analysis

Caspase-3 activity was measured as described previously [16]. The arbitrary fluorescence unit was corrected for the amount of protein determined using a protein assay kit (BioRad, Veenendaal, the Netherlands). Cell necrosis was determined by measuring LDH released into the supernatant of medium of cultured cells compared with LDH content from total cells. LDH activity was detected spectrophotometrically at 340 nm for 30 min.

Statistical Analysis

The data were expressed as mean ± standard error of the mean. *T*-test and one-way ANOVA were used for analysis. Results were considered statistically different when the *P* values were equal to or less than 0.05.

Results

Anti-inflammatory properties of *Ipomoea stolonifera* n-butanol extract and its purified components

Primary cultured hepatocytes were first challenged by a cytokine mixture (CM) to induce an inflammatory state in hepatocytes. Inducible Nitric Oxide Synthase (iNOS) mRNA was used as parameter for hepatocyte inflammation. The induction of iNOS was decreased significantly, but to different extents by BE-IS and its five components. In particular, curcumin (-98%) and hesperetin (-97%) dramatically reduced hepatic iNOS expression (Fig. 2A). Furthermore, iNOS expression was dose-dependently repressed by BE-IS up to 95% using the highest concentration of BE-IS (Fig. 2B). Interestingly, the mRNA levels of the anti-oxidant enzyme heme oxygenase-1 (HO-1) were increased by curcumin (9-fold), hesperetin (4-fold) and the raw extract BE-IS (5-31-fold) (Fig. 2C). Similar anti-inflammatory effects of BE-IS and its purified components were observed on the macrophage cell line RAW264.7 (Fig.2D).

***Ipomoea stolonifera* n-butanol extract and its purified components protect hepatocytes against bile acid-induced cell death**

The bile acid glycochenodeoxycholic acid (GCDCA) at 50 μmol/L induces apoptotic cell death of hepatocytes as determined by caspase-3 activity assay. Caspase-3 activation peaks 4-6 hrs after GCDCA exposure [17]. BE-IS and its purified components except esculetin demonstrated a profound reduction of caspase-3 activation induced by GCDCA (Fig. 3A). In particular, hesperetin proved to be very potent and the detailed investigation of this compound is described in Chapter 5 of this thesis. The anti-apoptotic effect of BE-IS was shown to be dose-dependent (Fig. 3B). The results obtained using the caspase-3 activity assay was confirmed using PARP-cleavage as another marker of apoptosis. Intact PARP (116 kD) is cleaved by caspase-3 into a lower molecular weight fragment of 89 kD. PARP-cleavage was almost completely prevented by BE-IS (Fig. 3C).

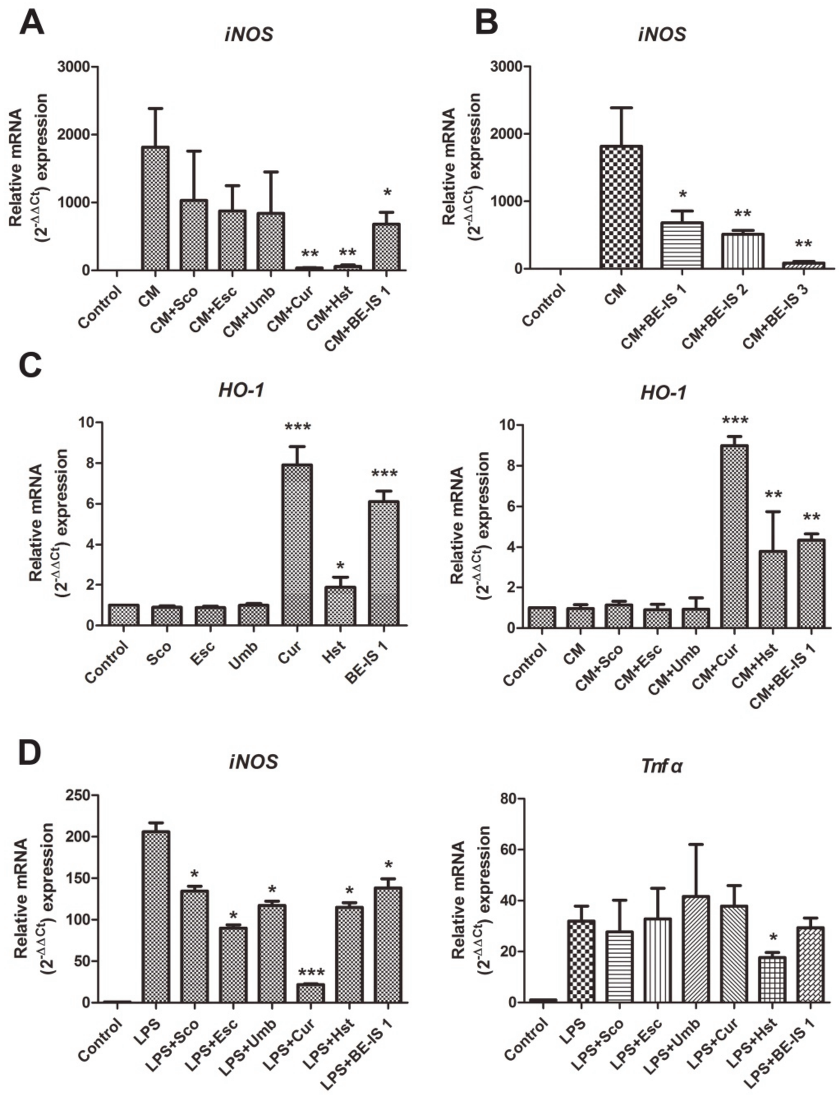

Figure 2. (A): The n-butanol extract of IS (BE-IS), curcumin and hesperetin reduce cytokine-mixture induced iNOS expression in hepatocytes. (B) BE-IS dose-dependently reduced CM-induced iNOS expression in hepatocytes. (C) BE-IS, hesperetin and curcumin induce HO-1 expression both in control and CM-exposed hepatocytes. (D) Anti-inflammatory effects of BE-IS and its purified components on RAW264.7 cells. mRNA levels are expressed as ($2^{-\Delta\Delta Ct}$) relative to control values. Doses of BE-IS 1, 2 and 3 used are 26.24, 52.48 and 104.96 µg/ml, respectively. The concentration of individual components is 50 µmol/L. Experiments were performed with hepatocytes from 4 different hepatocyte isolations. Scopoletin (Sco), Esculetin (Esc), Umbelliferone (Umb) Hesperetin (Hst), Curcumin (Cur) and n-butanol extract from *Ipomoea stolonifera* (BE-IS) (*P<0.05, ** <0.01, ***< 0,001 as compared to CM or LPS groups).

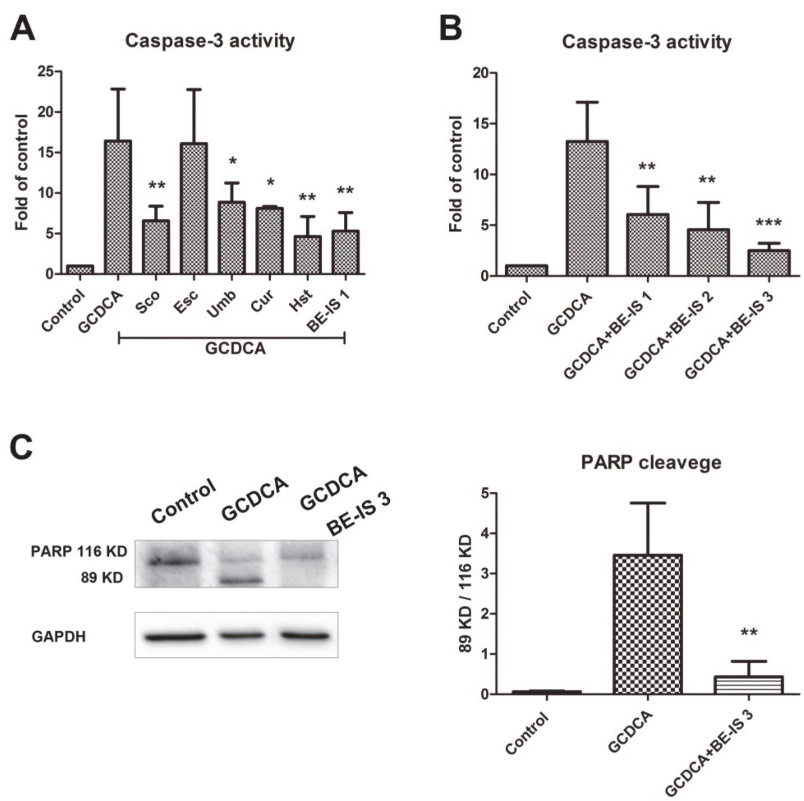

Figure 3. BE-IS and purified compounds protect hepatocytes against bile acid-induced cell death. (A) GCDCA-induced caspase-3 activity was decreased by all purified compounds except esculetin. (B) BE-IS dose-dependently inhibited GCDCA-induced caspase-3 activation. (C) BE-IS prevented GCDCA-induced PARP cleavage. Ratio 89kD/116kD was determined by densitometry and normalized for GAPDH values (right panel). GCDCA was used at 50 µmol/L and cells were harvested 4 hours after GCDCA exposure. Doses of BE-IS 1, 2, 3 used are 26.24, 52.48 and 104.96 µg/ml, respectively. The concentration of individual components is 50 µmol/L. Experiments were performed with hepatocytes from 4 different hepatocyte isolations. Scopoletin (Sco), Esculetin (Esc), Umbelliferone (Umb) Hesperetin (Hst), Curcumin (Cur) and n-butanol extract from *Ipomoea stolonifera* (BE-IS) (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to GCDCA).

The inhibition of apoptotic cell death by BE-IS and its components was not accompanied by an increase in necrotic cell death as determined by LDH release. In fact, the raw extract BE-IS tended to decrease LDH release in GCDCA-treated cells, indicating that BE-IS was truly protective and did not induce a shift from apoptotic to necrotic cell death (Fig. 4A). Likewise, BE-IS and its components did not induce cell death in cytokine mixture-treated hepatocytes, again indicating that the anti-inflammatory effect was not due to modulation of cell viability (Fig. 4B).

The protective effect of BE-IS is not dependent on the ERK1/2, p38 and PI3K pathways

In order to find out whether specific kinase pathways are involved in the protection of BE-IS, inhibitors of three kinases, known to be involved in the regulation of cell death (ERK1/2 and p38 MAP kinase and PI3-kinase) were added 30 min before GCDCA exposure. As depicted in Fig. 4C, none of the inhibitors had any effect on the anti-apoptotic action of BE-IS.

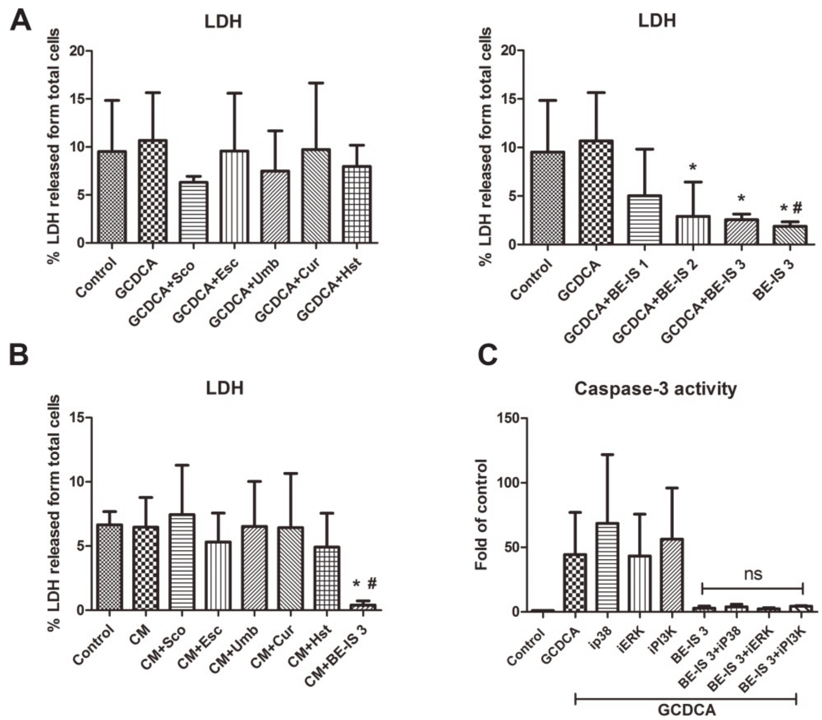

Figure 4. (A, B) GCDCA and cytokine mixture do not induce necrotic cell death as determined by LDH leakage and BE-IS and its constituents do not induce a shift from apoptotic towards necrotic cell death. In fact, BE-IS appears to reduce the low level of necrotic cell death even further. (C) The protective effect of BE-IS is not dependent on the activation of ERK1/2, p38 and PI3K pathways. Doses of BE-IS 1, 2, 3 used are 26.24, 52.48 and 104.96 µg/ml, respectively. The concentration of individual components is 50 µmol/L. Experiments were performed with hepatocytes from 4 different hepatocyte isolations. Scopoletin (Sco), Esculetin (Esc), Umbelliferone (Umb) Hesperetin (Hst), Curcumin (Cur) and n-butanol extract from *Ipomoea stolonifera* (BE-IS) (*$p<0.05$ compared to GCDCA, #$p<0.05$ compared to control).

Discussion

In chronic liver injury, a vicious cycle of inflammation, oxidative stress and hepatocyte cell death occurs: chronic liver inflammation leads oxidative stress and to hepatocyte cell death and, in turn, products of dead and/or damaged hepatocytes may drive inflammation. The continuous inflammation and wound healing response in chronic liver diseases may lead to liver fibrosis, cirrhosis and, ultimately, primary hepatocellular carcinoma [18]. In chronic liver diseases, hepatocytes may die from either apoptotic or necrotic cell death. Apoptotic or programmed cell death is executed by caspases that mediate the destruction of intracellular substrates leading to cell death [19-21]. Any effective intervention to treat chronic liver diseases must therefore be aimed at protecting the hepatocyte against apoptotic and/or necrotic death and reducing inflammation and accompanying generation of oxidative stress. Unfortunately, at present, no such interventions exist in clinical practice.

Natural products are increasingly considered in the treatment of chronic inflammatory conditions, including chronic liver diseases. In general, natural products are complex mixtures of bioactive compounds that usually act synergistically. Therefore, isolated compounds of these mixtures are frequently not or only partially effective compared to raw extracts of natural products. In previous research, we have investigated the n-butanol extract from *Ipomoea stolonifera* (BE-IS) in murine models of inflammation [10]. It was observed that BE-IS possessed potent anti-inflammatory activity in acute and chronic inflammation models [10]. Five active compounds have been purified from BE-IS: the coumarins scopoletin, esculetin and umbelliferone and the flavonoids hesperetin and curcumin. Although these compounds exhibit several bioactive properties [11-14], only few studies have been conducted to evaluate its hepatoprotective and anti-inflammatory effects in liver diseases.

In the present study we observed that the BE-IS and some of its purified components have strong cytoprotective and anti-inflammatory properties. The BE-IS significantly reduced apoptotic death of hepatocytes induced by the bile acid GCDCA. This effect was mimicked by the components hesperetin, scopoletin, curcumin and umbelliferone, but not by the coumarin esculetin. At present, it is not clear why esculetin differs from the other coumarins in terms of cytoprotection. Possible explanations include an intrinsic difference in anti-apoptotic potency between different members of the coumarin family or differences in uptake between different coumarins leading to different intracellular concentrations. Hesperetin appeared to be the most potent cytoprotectant and was able to reduce apoptotic cell death to the same extent as BE-IS. Of note, neither BE-IS nor its purified constituents caused a shift towards necrotic cell death, indicating that these compounds are truly protective. Hesperetin, together with curcumin also appeared to be the most powerful anti-inflammatory component of BE-IS, both in hepatocytes and in RAW264.7 macrophages. The RAW264.7 cell line is a mouse macrophage cell line that is often used to represent macrophages. In the liver, the largest populations of macrophages are the liver-specific Kupffer cells. Phenotypically, Kupffer cells differ from other macrophage populations, although they do have most macrophage functions like phagocytosis and LPS-induced cytokine (TNFα) production. Therefore, our findings with RAW264.7 macrophages need to be confirmed in Kupffer cells. Flavonoids like hesperetin are potent scavengers of reactive oxygen species. It remains to be elucidated whether the anti-inflammatory action of hesperetin is due to its ROS-scavenging potential or whether alternative mechanisms underlie its anti-inflammatory and cytoprotective actions. Interestingly, curcumin and hesperetin are also the only purified compounds that are able to induce the anti-oxidant gene heme oxygenase-1 (HO-1). HO-1 has been to be a protective and anti-oxidant gene

[22] and, of note, a reciprocal regulation of the inflammatory marker iNOS and HO-1 has been observed in intestinal epithelial cells previously [23]. Thus, hesperetin and curcumin could contribute to cell protection and reduced inflammation by both direct scavenging of ROS (as flavonoids) as well as indirectly by inducing HO-1. Previously, we have shown that increased expression of HO-1 is protective against ROS-induced cell death, in part via its product carbon monoxide [22]. It is important to note that the strongest inducer of HO-1 is the raw extract BE-IS, although we did not evaluate the combination of hesperetin and curcumin. In the current study we used the bile acid GCDCA as inducer of apoptotic cell death, but it is clear that the cytoprotective actions of BE-IS and the compounds hesperetin and curcumin need to be evaluated in models of ROS-induced cell death as well.

The protective and anti-inflammatory actions of curcumin need to be interpreted with caution, since the major obstacle of its use in clinical development is the oral bioavailability and increasing the dosage of curcumin has been prohibited due to its toxicity, including the induction of damage to nuclear and mitochondrial DNA and the inhibition of drug-metabolizing enzymes, such as cytochrome P450, glutathione-S-transferase and UDP glucuronosyl- transferase [24].

With regard to the mechanisms involved in the anti-inflammatory and cytoprotective actions of BE-IS and its constituents more research is needed. None of the inhibitors of known signal transduction kinases like p38, ERK1/2 and PI-3-Kinase modulated the actions of BE-IS and its constituents on inflammation and cell death. In hepatocytes, p38, ERK1/2 and PI-3-kinase all protect against bile acid-induced apoptosis [16]. This would indicate that these pathways are not involved in the protection by BE-IS, or that BE-IS and its constituents remove the immediate downstream apoptotic trigger of GCDCA, making it obsolete for hepatocytes to activate these protective pathways.

All in all, we show that BE-IS and its constituents hesperetin and curcumin display potent cytoprotective and anti-inflammatory actions. Since curcumin has been associated with toxicity in clinical trials, hesperetin could be the component of choice to evaluate in *in vivo* models of chronic liver diseases and eventually, in clinical trials. Therefore, we evaluated hesperetin in more detail in an *in vivo* model of inflammation, described in chapter 3 of this thesis.

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Chapter 3

The protective effect of the natural compound hesperetin against fulminant hepatitis *in vivo* and *in vitro*

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Abstract

Background: Liver diseases are mostly accompanied by inflammation and hepatocyte death. Therapeutic approaches targeting both hepatocyte injury and inflammation are not available. Natural compounds are considered as potential treatment for inflammatory liver diseases. Hesperetin, a flavonoid component of citrus fruits has been reported to have anti-inflammatory properties. The aim of this study was to evaluate the cytoprotective and anti-inflammatory properties of hesperetin both *in vitro* and in models of fulminant hepatitis.

Methods: Apoptotic cell death and inflammation were induced in primary cultures of rat hepatocytes by bile acids and cytokine mixture, respectively. Apoptosis was quantified by caspase-3 activity and necrosis by LDH release. The concanavalin A (ConA) and D-galactosamine/LPS (D-GalN/LPS) were used as models of fulminant hepatitis. Liver injury was assessed by ALT and AST levels, liver histology and TUNEL assay and inflammation by inducible Nitric Oxide Synthase (iNOS) expression.

Results: Hesperetin blocked bile acid-induced apoptosis and cytokine-induced inflammation in rat hepatocytes. Moreover, hesperetin improved liver histology and protected against hepatocyte injury in ConA- and D-GalN / LPS-induced fulminant hepatitis, as assessed by TUNEL assay and serum AST and ALT levels. Hesperetin also reduced expression of the inflammatory marker iNOS and the expression and serum levels of TNFα and IFN-γ, the main mediators of cell toxicity in fulminant hepatitis.

Conclusion: Hesperetin has anti-inflammatory and cytoprotective actions in models of acute liver toxicity. Hesperetin therefore has therapeutic potential for the treatment of inflammatory liver diseases accompanied by extensive hepatocyte injury, such as fulminant hepatitis.

Introduction

Hesperetin is a natural compound belonging to the flavanone class of flavonoids. It is the aglycone of hesperidin (β-7-rutinoside of hesperetin), a predominant flavonoid component of citrus fruits (Fig.1). It is now well accepted that a low consumption of fatty foods and an increased intake of fruit and vegetables will reduce the risk of some life-threatening diseases and maintain a good health status [1]. World-wide, the dieteray intake of citrus fruit products, and hence flavanones, is increasing every year [2]. In Western countries, the intake of hesperetin is largely dependent on dietary habits [3], whereas hesperetin is also known as a major active ingredient in the Chinese traditional medicinal herb Chenpi [4]. Because of the reported bioactivities, extensive research has been performed on hesperidin and hesperetin in various experimental models. These bioactivities include antioxidant, anti-inflammatory and anticarcinogenic effects [5, 6].

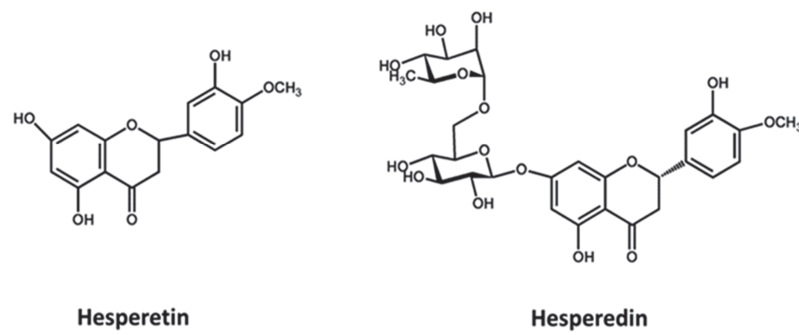

Figure 1. Structures of hesperitin and hesperidin

Most liver diseases are accompanied by inflammation and oxidative stress, regardless of the etiology of the underlying disorder. Mild and time-restricted hepatic inflammation could be considered beneficial in the restoration of tissue homeostasis, e.g. by eliminating invading pathogenic organisms and damaged or dead cells. However, excessive and uncontrolled inflammation leads to massive loss of hepatocytes as a result of apoptosis and/or necrosis [7] irreversible damage to the liver parenchyma and loss of liver function [8]. Loss of hepatocytes and loss of liver function occurs in many liver pathologies, including fulminant hepatitis, reperfusion injury, (non-)alcoholic liver diseases, cholestasis and viral hepatitis. All these conditions demonstrate high morbidity and mortality and liver transplantation is often the only life-saving treatment [9, 10]. The management of acute and chronic inflammatory liver disease is still a challenge to modern drug development, because there are currently no effective treatments that improve liver function and/or regenerate or protect hepatic cells [11]. Therefore, there is an urgent need for novel therapeutic approaches that prevent liver injury via protection against hepatocyte cell death. In particular, the potential of herbal and dietary supplements, like hesperetin has been largely unexplored in this regard.

Although hesperidin possesses a wide range of biological activities, including hepatoprotective properties in liver injury [12-14], its aglycone-derivative hesperetin has stronger bioactivity as a result of more efficient absorption from the intestine than hesperidin [15-17], [18]. Existing studies are mainly focused on one specific *in vivo* or *in vitro* model [19, 20], but comprehensive reports on the effectiveness of different doses of orally administered hesperetin in multiple models of fulminant hepatitis are lacking. In viral and autoimmune hepatitis, activation of T-cells and macrophages is the initial event [21]. Experimental liver injury models were established that resemble fulminant human hepatitis, including TNFα- and IFN-γ-dependent inflammatory liver injury models that allow the evaluation of hepatoprotective interventions, including medicinal plant components. In our study, immune-mediated liver injury was induced by the T-cell mitogenic plant lectin concanavalin A (Con A). Liver injury in this model is dependent on both macrophage-derived TNFα and T-cell-derived IFN-γ. In this model the expression of various cytokines is strongly induced, including IFN-γ, IL-4, and IL-2 [22]. As a second model, we used an inflammation-induced model of fulminant hepatitis. Endotoxins like LPS are known as strong stimulators of macrophages, including Kupffer cells. TNFα alone does not induce hepatocyte cell death. However, when hepatocytes are simultaneously sensitized with D-galactosamine (D-GalN), preventing hepatocyte transcription, LPS-induced TNFα becomes extremely hepatotoxic, because of massive apoptosis of hepatocytes [23].

The aim of the present study was to investigate the hepatoprotective and anti-inflammatory properties of hesperetin in acute liver injury. We demonstrate that hesperetin is anti-inflammatory and cytoprotective, in part by repression of IFN-γ expression in T-cell-mediated hepatitis and by repression of TNFα expression in the TNFα-dependent D-GalN/LPS model of liver injury.

Methods

Animals

Male Wistar rats (220-250g) were purchased from Harlan (Zeist, the Netherlands) Experiments were performed after approval by and following the guidelines of the local Committee for Care and Use of Laboratory Animals of the University of Groningen. 6 week-old male BALB/c mice (20-22g) were obtained from Hunan SJA Laboratory Animal Co. Ltd (Changsha, China NO. 43004700009427). All animals received humane care according to the legal requirements and guidelines approved by the ethics committee for the animal facility of Shantou University Medical College. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [24, 25]. All animals were maintained under controlled conditions and had free access to standard laboratory chow and water.

Hepatocyte isolation

Hepatocytes were isolated from Wistar rat by a two-step collagenase perfusion procedure as described previously [26]. Cell viability was determined by trypan blue staining and was more than 85%. 112,500 cells per cm^2 were plated on Vitrogen®-coated plates in William's E medium (Life Technologies Ltd; Breda, The Netherlands) supplemented with 50 µgmL gentamycin (Life Technologies Ltd) and penicillin-streptomycin-fungizone (Lonza, Verviers, Belgium). During the attachment period (4 h) 50 nmol L^{-1} dexamethasone (Sigma, St Louis, USA) and 5% fetal calf serum (Life Technologies Ltd) were added to the medium. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2.

***In vitro* studies**

Experiments were started after the attachment period of 4 hours. Monolayers of cultured primary hepatocytes were treated with different concentrations of hesperetin (10, 25, 50 µmol L^{-1}) to analyze the effect on cytokine mixture (CM: 20 ng ml^{-1} mTNFα, 10 ng ml^{-1} hIL-1β and 10 ng ml^{-1} rIFN-γ)-induced inflammation for 6 h. As *in vitro* model of cell death we used glycochenodeoxycholic acid (GCDCA: 50 µmol L^{-1})-induced cell death for 4 h. GCDCA-induced cell death is independent of any inflammation and induces mainly apoptosis [27]. Signal transduction pathways were inhibited using 10 µmol L^{-1} of the ERK1/2 inhibitor U0126 (Promega, Madison, USA), 10 µmol L^{-1} of the p38 inhibitor SB 203580 (Calbiochem, San Diego, CA, USA), 50 µmol L^{-1} of the PI3 kinase inhibitor LY 294002 (Calbiochem, San Diego, CA, USA). All inhibitors and receptor antagonists were added to the cultured hepatocytes 30 minutes prior to the apoptotic or inflammatory stimuli. Every experimental condition was performed in triplicate wells and each experiment was repeated at least four times using hepatocytes from different rats. Cells were harvested at the indicated time points using lysis buffer for protein assay or TriZol reagent for RNA isolation.

Liver injury experimental models

After 7 days of adjusting, the animals were randomly divided into 10 experimental groups.

Control group (n=8): These animals were treated with the equivalent volume of PBS as used for the administration of Con A and D-GalN/LPS.

Control hesperetin group (n=8): The mice were administered hesperetin 400 mg kg^{-1} p.o in 0.5% CMC-Na solution for 10 days.

Con A group (n=15): The animals were administered the same volume of CMC-Na as used for administration of hesperetin for 10 days and were challenged with Con A (i.v.15mg kg^{-1}).

Con A + hesperetin groups: The animals received various doses of hesperetin (100, 200, 400 mg kg^{-1}) orally for 10 days before Con A injection (each group n=15).

D-GalN/LPS group (n=15): The animals were administered CMC-Na for 10 days and intraperitoneally injected with D-GalN (700 mg kg^{-1})/LPS (5 μg kg^{-1}).

D-GalN/LPS + hesperetin groups: Three doses of hesperetin (100, 200, 400 mg kg^{-1}) were given to mice once per day for 10 days. D-GalN (700mg kg^{-1})/LPS (5 μg kg^{-1}) were administered intraperitoneally (each group n=15).

Con A from *Canavalia ensiformis* (Jack bean) Type IV, lyophilized powder (Sigma C2010), LPS (*Escherichia coli* 055:B5; Sigma L2880) and D-(+)-Galactosamine hydrochloride (Sigma G1639) were diluted in sterile endotoxin-free PBS. Mice were pretreated with hesperetin (3', 5, 7-Trihydroxy-4'-methoxyflavanone, Afar Aesar B20528) dissolved in sterile PBS containing 0.5% sodium carboxymethylcellulose CMC-Na (Aladdin) orally for 10 days once per day. Con A (15 mg kg^{-1}) was injected intravenously, and D-GalN (700 mg kg^{-1})/LPS (5 μg kg^{-1}) was administered intraperitoneally. Animals were sacrificed 8 h after the challenge. Blood was collected and the whole liver was harvested. The left lateral lobe was used for routine histology and the remaining lobes were frozen in aliquots in liquid nitrogen and then stored at -80°C for RNA isolation and protein assays. Serum was separated by centrifugation (15 min at 4,000 rpm) and used for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination and ELISA assays.

Routine histological analysis

Liver tissue was fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned into 4-μm sections. The tissue paraffin sections were deparaffinized, rehydrated following routine methods, and stained with hematoxylin-eosin (HE).

TUNEL assay

Apoptotic cell death was determined on paraffin-embedded sections of liver tissue by the presence of free 3′-hydroxy groups by TUNEL assay using DeadEnd™ Fluorometric TUNEL System (Promega, Beijing, China). The assays were performed as recommended by the manufacturer. For each liver tissue section, the number of TUNEL-positive cells in 3 random 20x-objective high-powered fields (containing at least one portal triad and central vein each) was counted by an investigator blinded with respect to treatment group using an Olympus IX81 microscope (Olympus, Japan).

Serum biochemical parameters

Activities of serum aminotransferases (ALT, AST) and total bilirubin (TBil) were determined by Bio-sinew kits (Chengdu, China) on Automatic Chemistry Analyzer (Accute TBA-40FR, Toshiba Medical Systems Corporation, Japan). Cytokine serum concentrations were assayed for murine TNFα, IL-4 (4A Biotech, Beijing, China), IFN-γ, (Boster, Wuhan, China), IL-6, and IL-10 (Bangyi, Shanghai, China) by enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (PCR)

RNA was isolated from mouse liver tissue using TriZol Reagent (TaKaRa, Japan). After using PrimeScript TM RT reagent Kit with gDNA Eraser (TaKaRa, Japan), quantitative real-time PCR (qRT-PCR) of liver tissue was performed using SYBR Green reagent (TaKaRa, Japan) on PCR detection system (ABI 7500, Applied Biosystems, USA). Rat mRNA from hepatocytes was isolated using Tri-reagent (Sigma-Aldrich), and assayed with TaqMan control reagents (ABI PRISM 7700, Applied Biosystems, The Netherlands). Mice primers and rat primers and probes are described in Table 1. Gene expression *in vivo* was normalized with respect to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and 18S for hepatocytes shown relative to control values.

Western blot analysis

Protein extracts from liver were prepared by homogenization in RIPA lysis buffer (P0013B Beyotime, Jiangsu, China). 50 microgram of protein from each sample was separated by SDS-PAGE and transferred to nitrocellulose filter membranes. Following blocking, membranes were probed with primary antibodies: mouse anti-iNOS rabbit monoclonal antibody 1:2,000 (Cell Signaling Technology, USA), mouse anti-phospho-SAPK/JNK (Thr183/Tyr185) rabbit monoclonal antibody 1:2,000 (Cell Signaling Technology, USA), anti-SAPK/JNK rabbit monoclonal antibody 1:3,000 (Cell Signaling Technology, USA). Mouse anti-GAPDH monoclonal antibody 1:3,000 (ZSGB-BIO, Beijing). All primary antibody incubations were overnight at 4°C, followed by detection using HRP-conjugated secondary mouse antibody 1:60,000 and rabbit antibody 1:80,000 (ZSGB-BIO, Beijing) at room temperature for 1 h. Total hepatocyte lysates were analyzed for Poly (ADP-ribose) polymerase (PARP) cleavage (1:1,000 Cell Signaling Technology, Beverly, Massachusetts, USA).

Analysis of hepatocyte apoptosis and necrosis

Measurement of caspase-3 activity was as described previously [26]. The arbitrary fluorescence unit was corrected for the amount of protein using BioRad protein assay kit. Cell necrosis was determined by measuring LDH release according to standard laboratory protocol. Briefly, 100 µl medium was loaded in 96-well plates followed by addition of pyruvate and NADH. LDH activity was detected by absorbance at 340 nm for 30 min. The linear portion of the kinetic curve was calculated compared to a standard curve.

Data and statistical analysis

The data were expressed as mean ± standard error of the mean. One-way ANOVA and *t*-test were used to analyze the results. Results were considered statistically different when the *P* values were equal to or less than 0.05.

Results

Hesperetin has anti-inflammatory and cytoprotective effects on rat hepatocytes

The bile acid glycochenodeoxycholic acid (GCDCA; 50 $\mu molL^{-1}$) induces apoptosis in primary rat hepatocytes with caspase-3 activity peaking after 4 h exposure [23, 28]. This model of apoptosis was used because it is not accompanied by inflammation and GCDCA does not activate NF-κB and therefore, any cytoprotective effect of hesperetin is independent of an effect on inflammation. Hesperetin was added 30 min prior to GCDCA and the effect on GCDCA-induced caspase-3 activity was investigated 4 hours after GCDCA exposure. Hesperetin dose-dependently reduced GCDCA-induced caspase-3 activity in cultured primary rat hepatocytes (Fig.2 A). Maximum inhibition (-80%) was observed at 50 $\mu mol\ L^{-1}$ hesperetin. Therefore, this concentration of hesperetin was used in subsequent analyses. Hesperetin alone, at 50 $\mu molL^{-1}$, did not modulate caspase-3 activity. In concordance, GCDCA induced cleavage of the caspase-3 substrate PARP, which was effectively inhibited by hesperetin (Fig. 2B). The anti-apoptotic effect of hesperetin was not accompanied by an increase in necrotic cell death as neither GCDCA, nor hesperetin nor the combination GCDCA and hesperetin induced LDH release from hepatocytes (Fig. 2C). To investigate the role of specific signal transduction pathways in the protective effect of hesperetin, we used several inhibitors of MAP Kinases and PI3K. Importantly, the protective effect of hesperetin against GCDCA-induced apoptosis was not abolished by inhibition of either ERK, p38 or PI3K (Fig. 2D). Hesperetin also dose-dependently reduced CM-induced *Nos2* (iNOS) expression in hepatocytes (-41%, -63% and -83% at 10, 25 and 50 $\mu molL^{-1}$, respectively) indicating that hesperetin also has potent anti-inflammatory properties (Fig. 2E). Interestingly, hesperetin induced expression of the anti-oxidant gene *Hmox1* (HO-1) about 4-fold compared to cytokine mixture alone (Fig. 2E).

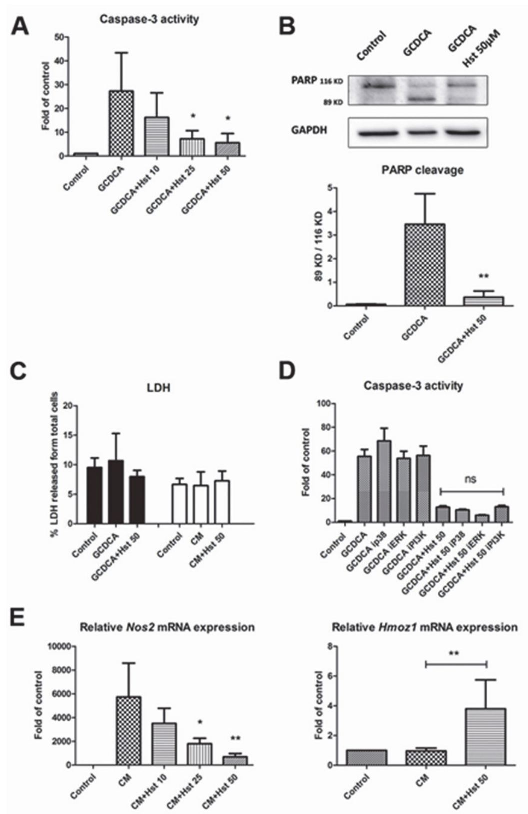

Figure 2. Hesperetin has cytoprotective and anti-inflammatory effects on primary rat hepatocytes. (A) Hesperetin reduces GCDCA-induced caspase-3 activation (GCDCA: 50 μmol L-1). (B) Hesperetin prevents cleavage of PARP as assessed by Western blot. (C) GCDCA and cytokine mixture (CM) do not induce necrotic death of hepatocytes. The inhibitory effect of hesperetin on GCDCA-induced apoptosis and CM-induced inflammation is not accompanied by an increase of necrosis as assessed by LDH leakage in supernatant of cultured hepatocytes. LDH release is expressed as % of total LDH content of hepatocytes. (D) The protective effect of hesperetin is not abolished upon inhibition of the p38 and ERK MAP kinases and the PI3K pathway. (E) Hesperetin attenuates the inflammatory response of hepatocytes as assessed by iNOS mRNA determination by qPCR; All experiments were performed in duplicate wells and each experiment was repeated using hepatocytes from 5 different isolations; Values are mean ± SD, ns indicates $P>0.05$ not significant, $^*P<0.05$ compared with GCDCA or CM.

Hesperetin attenuates Con A-mediated hepatitis

To translate our *in vitro* findings into an *in vivo* model, we first tested the effect of hesperetin on Con A-induced liver damage. The morphological observations are shown in Fig. 3A. Macroscopically, hesperetin reversed the dark surface color of livers with passive congestion induced by Con A (Fig. 3A). Microscopically, areas of active hepatocellular degeneration and necrosis are observed, presenting single or multiple foci of pale-staining groups of hepatocytes. In addition, congestion and inflammation (infiltration of mononuclear cells along with neutrophils) of pericentral areas is observed (Fig. 3B). Due to the limited period after Con A challenge, a normal

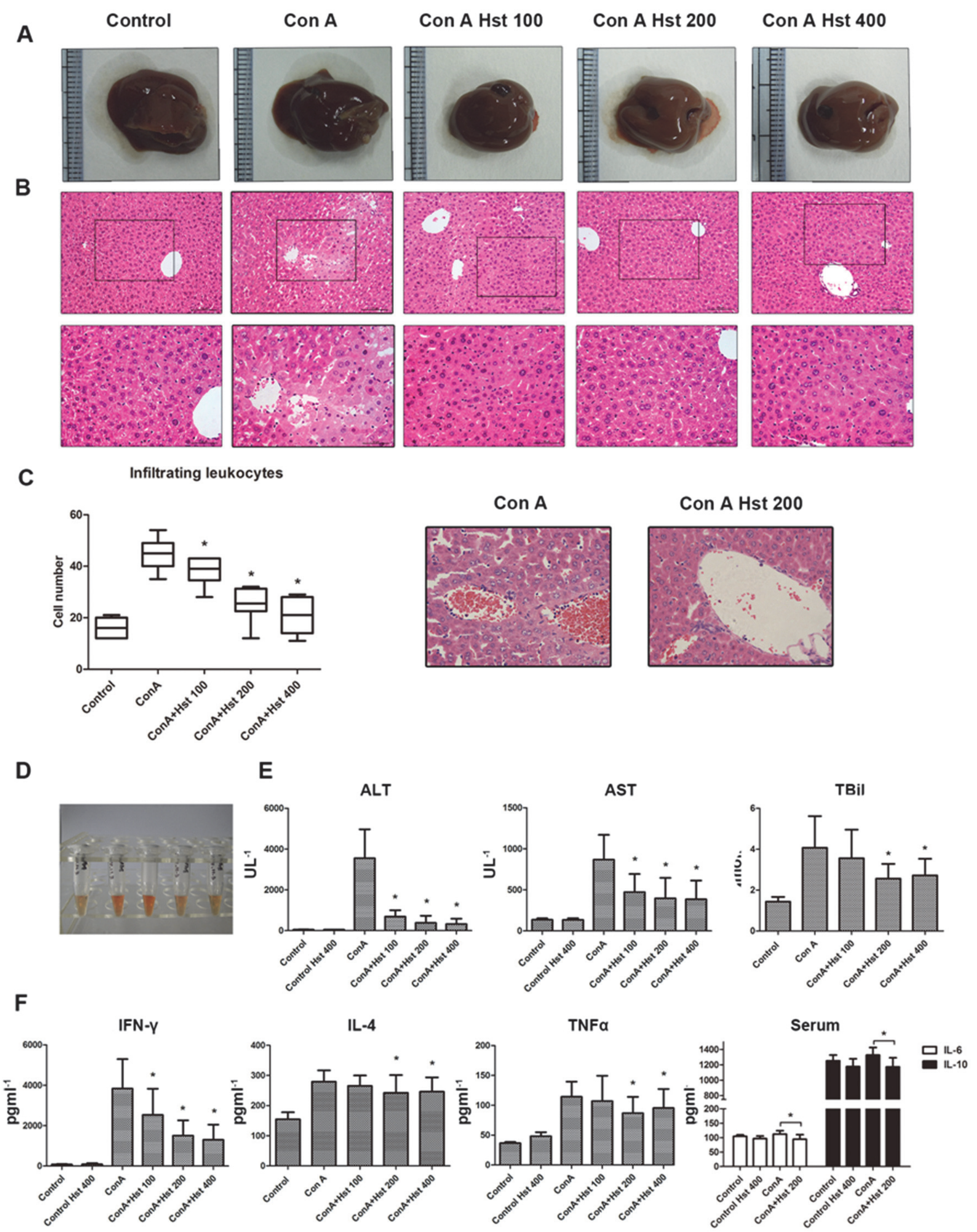

Figure 3. Hesperetin dose-dependently protects against Con A-mediated fulminant hepatitis. (A) Macroscopic appearance of livers indicating a beneficial effect of hesperetin to the dark surface of liver with passive congestion. (B) Hematoxylin-eosin staining of liver sections: in Con A hepatitis it shows hepatocellular degeneration (cloudy swelling), eosinophilic focus of cellular alteration with pale pink cytoplasm, condensed hypereosinophilic cytoplasm and shrunken nuclear occur spontaneously with one or two affected hepatocytes (arrows) and infiltration of inflammatory cells (mononuclear: lymphocytes and macrophages) (arrowheads), which are significantly reduced by Hst. Magnification 200X (upper panel), 400X (lower panel). (C) Quantitation of infiltrating leukocytes, represented as average number of at least 10 individual liver sections in Con A and Con A + Hesperetin groups (8

liver sections in Control group). Magnification of measurement area is 400X. (D) Serum samples show increased total bilirubin levels in Con A-treated animals, which is (partially) reversed by hesperetin. Samples represent (from left to right): Control, Con A, Con A and increasing doses of hesperetin. (E) Serum markers of liver injury, AST, ALT and total bilirubin, are significantly induced in Con A hepatitis. Hst attenuates the rise in ALT, AST and TBil levels. (F) Serum levels of cytokines as assessed by ELISA demonstrate a significant rise of IFN-γ and TNFα in Con A hepatitis, which is reduced by Hst. Values are mean ± SD; Control group and Control Hesperetin group n=8, Con A and Con A + Hesperetin groups n=15, *P<0.05 compare with Con A.

liver structure is still retained. These microscopic abnormalities were prevented or reversed by hesperetin (Fig. 3B). In fact, the histology of Con A-treated groups that received 200 and 400 mg kg^{-1} hesperetin was similar to normal liver histology. The number of infiltrating leukocytes in liver tissues of mice with Con A induced fulminant hepatitis are significantly decreased by hesperetin, especially in portal venous areas (Fig.3C). Serum ALT and AST were increased 75- and 6-fold, respectively after Con A treatment. Hesperetin pretreatment dose-dependently attenuated the Con A-induced increase of serum AST, ALT and TBil (Fig. 3D, E). The protective effect of hesperetin was paralleled by a significant dose-dependent decrease of serum IFN-γ levels (Fig. 3F). Con A treatment also increased TNFα and interleukin-4 (IL-4) (Fig. 3F). Co-treatment with hesperetin reduced the serum levels of these cytokines as well, albeit to a lower extent compared to IFN-γ. IL-6 and IL-10 serum levels were hardly affected by Con A treatment and hesperetin has only minor effects on the serum levels of those cytokines (Fig. 3F).

Hesperetin attenuates Con A-induced hepatocyte apoptosis and hepatic *Nos2* (iNOS) expression

In addition to necrosis, Con A induced hepatocyte apoptosis, as detected by TUNEL staining and caspase-3 activity assay, which was markedly reduced by co-treatment with hesperetin at 200 mg kg^{-1} (Fig. 4A, Fig.5). Furthermore, hesperetin suppressed Con A-induced iNOS protein and mRNA (*Nos2*) expression (Fig. 4B, C). Con A treatment induced the hepatic mRNA levels of several inflammatory and T-cell-derived cytokines, such as TNFα, IFN-γ, Il-6, Il-10, Il-4 and Il-1β. Expression of all these cytokines was reduced by hesperetin co-treatment (Fig. 4C) and these results generally paralleled the serum levels of these cytokines (Fig. 3E).

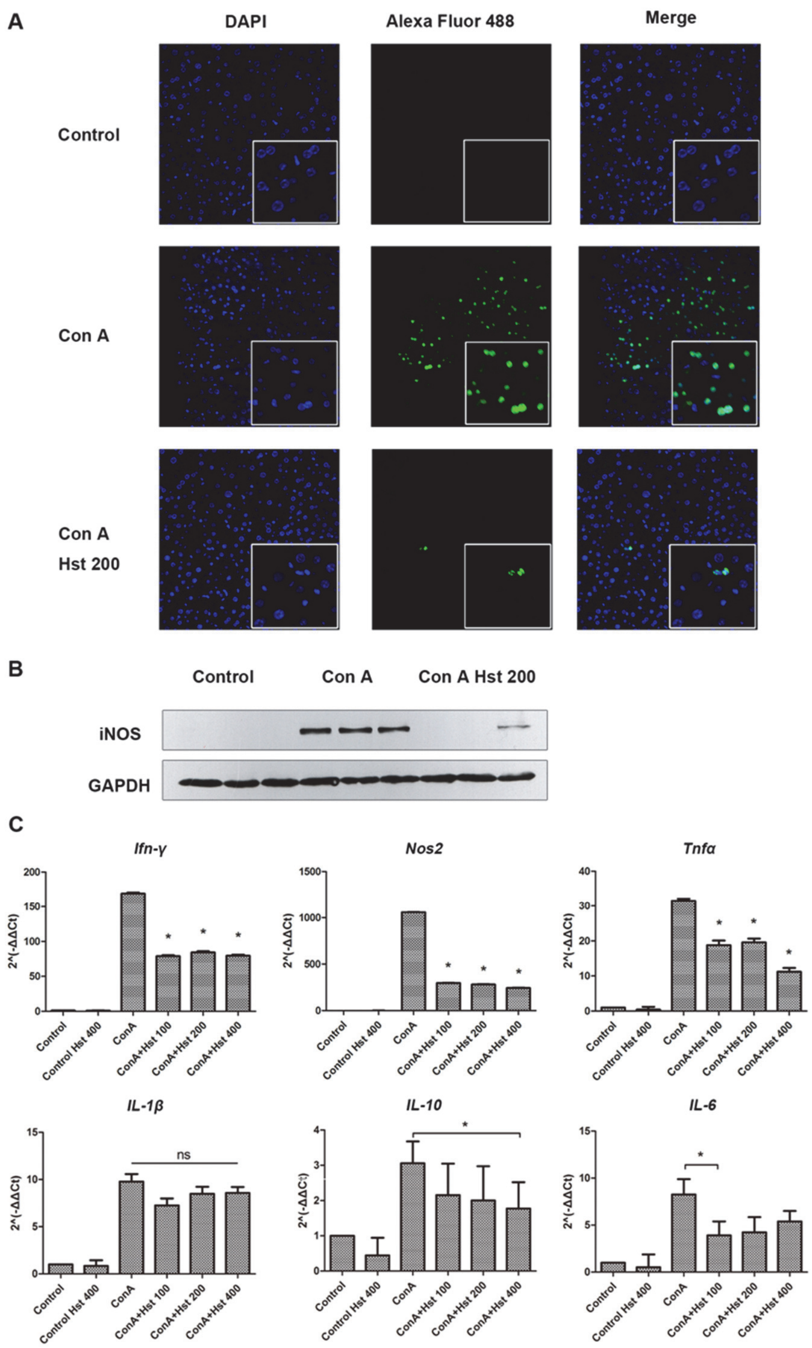

Figure 4. Apoptotic cell death and inflammation in Con A induced fulminant hepatitis are attenuated by hesperetin. (A) Apoptosis was assessed by TUNEL assay and visualized by Alexa Fluor 488. Hesperetin significantly reduced the number of TUNEL-positive nuclei in Con A hepatitis; (B) Hst attenuated Con A-induced inflammation as assessed by iNOS Western blot analysis. GAPDH was used as a loading control. (C) Expression of cytokines in liver tissue was determined by qPCR and expressed as fold increase compared to control. Con A induced the expression of inflammatory and T-cell derived cytokines. The induction of all cytokines, except Il-1β was attenuated by hesperetin. Values are mean ± SD; Control group n=8, Con A and Con A + Hesperetin groups n=15. *P<0.05 compare with Con A.

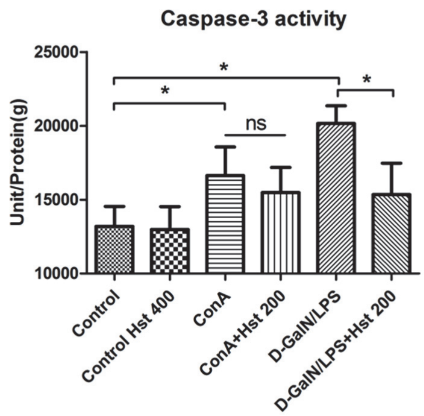

Figure 5. Hesperetin reduces caspase-3 activity in fulminant hepatitis. Hesperetin attenuates caspase-3 activity in fulminant hepatitis. Hesperetin (Hst) was administered to animals at 200 mg kg-1. D-GalN/LPS and to a lesser extent Con A, induced an increase in caspase-3 activity, which was significantly attenuated by Hst in the D-GalN/LPS model. Values are mean ± SD *P<0.05.

3

Hesperetin protects mice from D-GalN/LPS induced liver injury

Another well-characterized murine model of acute liver failure is LPS administration to D-GalN-sensitized mice. In this model, hesperetin showed a protective effect as demonstrated by the reversal of the darker surface of the livers as blood-filled on macroscopic appearance (Fig. 6A). In addition, hesperetin improved histology in HE-stained tissue sections. Hesperetin decreased the extent of piecemeal necrosis around central veins and the loss of normal morphology. Hesperetin co-treatment also decreased the occurrence of apoptotic bodies, hydropic degeneration, nuclear fragments, autolysis and hemorrhage (Fig. 6B). The number of leukocytes infiltrated in liver tissue of mice with D-GalN/LPS induced fulminant hepatitis are significantly decreased by hesperetin (Fig.6C). Serum markers of liver injury (AST, ALT) were reduced by hesperetin co-treatment, but hesperetin did not attenuate the increased levels of TBil (Fig. 6D, E). Remarkably, in this model hesperetin was most protective at 200 mg kg-1. Treatment with both 100 mg kg-1 as well as 400 mg kg-1 hesperetin resulted in less complete or no protection at all compared to 200 mg kg-1 hesperetin (Fig. 6A,B,C,D,E). ELISA assay on serum samples demonstrated a clear reduction of LPS/D-GalN-induced TNFα level by hesperetin at 200 mg kg-1, but not at 100 or 400 mg kg-1. The effect of hesperetin on the serum levels of other cytokines (IL-6, IL-10) was less conclusive (Fig. 6F).

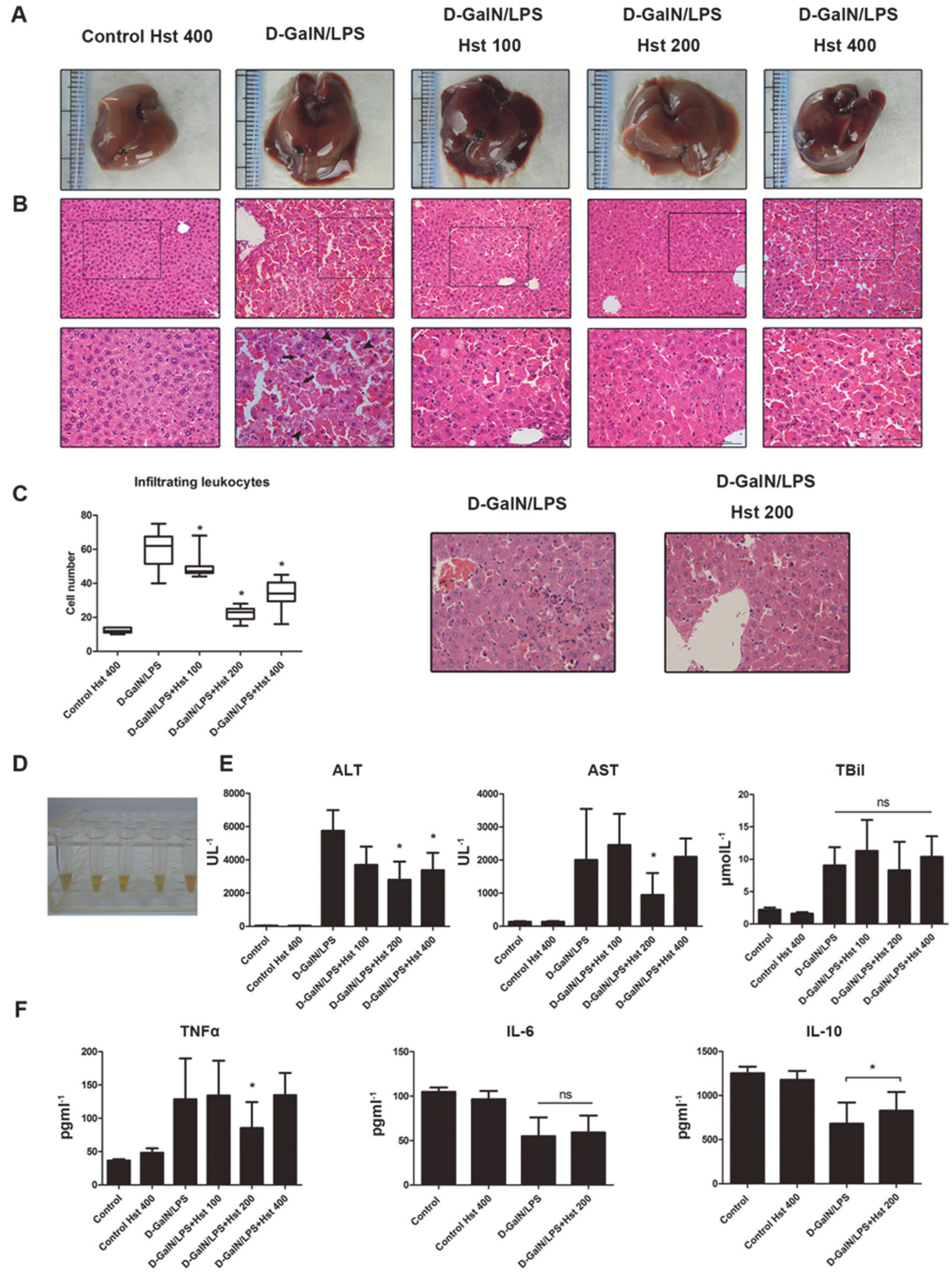

Figure 6. Hesperetin protect mice from D-GalN/LPS induced fulminant hepatitis. (A) Macroscopic appearance of livers. (B) Hematoxylin-eosin staining of liver sections: In D-GalN/LPS-induced fulminant hepatitis there is pale eosinophilic staining, absence of nuclear detail, nuclear fragmentation (arrows), patchy areas of blood and abundant apoptotic hepatocytes (arrowheads). Magnification 200X (upper panel), 400X (lower panel). (C) Quantitation of infiltrating leukocytes, represented as average number of at least 10 individual liver sections in D-GalN/LPS and D-GalN/LPS + Hesperetin

groups (8 liver sections in Control group). Magnification of measurement area is 400X. (D) Serum samples show hyperbilirubinemia in D-GalN/LPS -treated animals, which is not reversed by hesperetin. Samples represent (from left to right): Control, Hesperetin, D-GalN/LPS, D-GalN/LPS and increasing doses of hesperetin. (E) Serum markers of liver injury, AST, ALT and total bilirubin (TBil) are significantly induced in D-GalN/LPS hepatitis. Hst attenuates the rise in ALT and AST levels but not in TBil levels. (F) Serum levels of inflammatory cytokines as assessed by ELISA. D-GalN/LPS induces a strong increase in TNF serum levels which is attenuated by hesperetin. Values are mean ± SD; Control group and Control Hesperetin group n=8, D-GalN/LPS and D-GalN/LPS + Hesperetin groups n=15. ns: not significant, *P<0.05 compare with D-GalN/LPS.

Hesperetin inhibits pro-apoptotic JNK activation and inflammation in D-GalN/LPS-challenged mice

In addition to necrosis, D-GalN/LPS induced significant apoptosis as determined by TUNEL assay and caspase-3 activity assay. Hesperetin at 200 mg kg^{-1} reduced the number of TUNEL-positive hepatocyte nuclei and caspase-3 activity in liver tissue (Fig. 5 and Fig. 7A). Moreover, the D-GalN/LPS-induced activation of pro-apoptotic JNK was reduced by 49% by hesperetin treatment (200 mg kg^{-1}) (Fig. 7B). D-GalN/LPS-induced acute liver failure is characterized by a strong inflammatory response. Indeed, hepatic mRNA levels of the inflammatory cytokines TNFα, IL-1β and IL-6 were all strongly induced after D-GalN/LPS treatment and this induction was reduced by hesperetin by -61%, -68% and -75%, respectively (Fig. 7C). To better characterize the molecular mechanisms underlying the protection of hesperetin, selective genes were determined by qPCR. Both FasL and TLR-4 expression were increased in the D-GalN/LPS model and this increased expression was attenuated by hesperetin at 200 mg kg^{-1}. The endotoxin receptor TLR-4 and FasL are mainly expressed on macrophages in the liver, including Kupffer cells and immune cells and reflect increased inflammation. The effects of hesperetin on the expression of these markers indicate decreased inflammation (Fig. 7C).

To determine whether hesperetin is also effective when administered after the challenge (therapeutic effect), we performed a proof of concept study in which hesperetin was given 1 hour and 3.5 hours after D-Gal/LPS challenge. In this pilot experiment, using Kunming mice, hesperetin significantly reduced AST and ALT levels compared to treatment with D-GalN/LPS alone (data not shown).

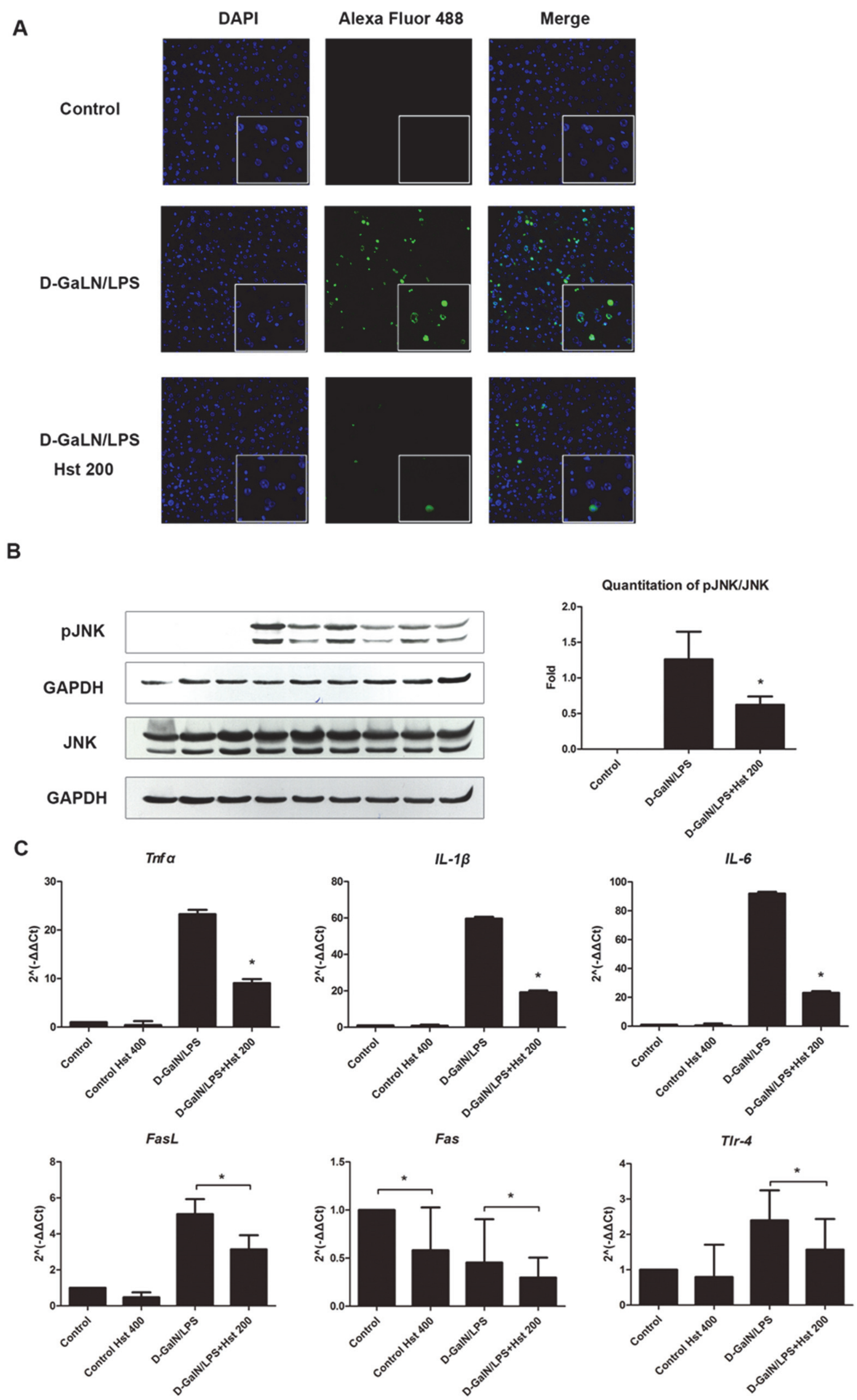

Figure 7. Apoptotic cell death and inflammation in D-GalN/LPS-induced fulminant hepatitis are attenuated by hesperetin. (A) Apoptosis was assessed by TUNEL assay and visualized by Alexa Fluor 488. Hesperetin significantly reduced the number of TUNEL-positive nuclei in D-GalN/LPS induced hepatitis; (B) Hesperetin attenuated D-GalN/LPS-induced activation of JNK as assessed by Western

blot analysis for phospho-JNK. Total JNK and GAPDH were used as reference proteins. Right panel shows the quantitation of pJNK/JNK. (C) Expression of cytokines in liver tissue was determined by qRT PCR and expressed as fold increase compared to control. The inflammatory cytokines TNFα, IL-1β and IL-6 are induced in D-GalN/LPS hepatitis and this induction is attenuated by hesperetin. Furthermore, expression of FasL and TLR-4, mainly expressed on inflammatory and immune cells, is increased in D-GalN/LPS hepatitis and this increase is attenuated by hesperetin. Values are mean ± SD; Control group and Control Hesperetin group n=8, D-GalN/LPS and D-GalN/LPS + Hesperetin groups n=15, *P<0.05 compare with D-GalN/LPS.

Discussion

Use of herbal preparations can be traced back over centuries and has been described in ancient Egypt, China, India and Sumeria [29]. It is an important component of complementary and alternative medical therapies, together with dietary supplements [30] and many people consider herbal remedies as natural and free of side-effects and beneficial for health maintenance [31, 32]. Therefore, the popularity of complementary and alternative medical therapies is increasing every year. Hesperetin is a bioactive flavanone in citrus fruits and its consumption is increasing worldwide [2], although a systematic evaluation of orally administered hesperetin for the treatment of liver diseases, in particular fulminant hepatitis is still lacking. In the present study, we investigated the protective effect of hesperetin on hepatic injury using both *in vivo* and *in vitro* models. We demonstrated that hesperetin protected hepatocytes against apoptosis in an inflammation-independent model of bile acid-induced apoptosis and reduced markers of NF-κB activation like iNOS, indicative of inflammatory signaling in both macrophages as well as hepatocytes. Furthermore, hesperetin proved to be protective in two models of fulminant hepatitis, reducing both inflammation and cell injury.

Virtually all liver diseases are accompanied by inflammation. Mild and time-restricted hepatic inflammation contributes to the restoration of tissue homeostasis. In contrast, continuous and uncontrolled inflammation leads to massive loss of hepatocytes and loss of liver function [8] as a result of apoptosis or necrosis [33]. Inflammation and hepatocyte death results in a vicious cycle: inflammation drives hepatocyte injury and death via increased generation of apoptotic cytokines and reactive oxygen species by inflammatory cells, whereas debris of injured and dead hepatocytes drive inflammation. Because of this vicious cycle, any treatment for liver injury should ideally combine anti-inflammatory actions on inflammatory cells and cytoprotective actions on hepatocytes. Natural products are increasingly considered in the treatment of inflammatory diseases, such as rheumatoid arthritis. Recently, we described the anti-inflammatory activity of the n-butanol extract from *Ipomoea stolonifera* in acute models of inflammation [34]. The n-butanol extract of *Ipomoea stolonifera* contains five major components, including hesperetin. In preliminary *in vitro* experiments, hesperetin demonstrated to have potent anti-inflammatory activity. Therefore, hesperetin was chosen to be evaluated in this study for the treatment of inflammatory liver diseases.

Hesperetin protected hepatocytes against bile acid (GCDCA)-induced apoptosis. GCDCA-induced apoptosis is dependent on the mitochondrial pathway and is accompanied by the activation of mitochondria-specific caspase-9. Importantly, GCDCA does not induce an inflammatory response in hepatocytes, demonstrating that the cytoprotective effect of hesperetin is independent of any anti-inflammatory effect of hesperetin. Furthermore, the protective effect of hesperetin was independent of ERK MAP kinase, p38 MAP kinase and PI3K signaling. Previously, we reported on the protective effect of the therapeutic bile acid tauroursodeoxycholic acid (TUDCA) on GCDCA-induced apoptosis, which is dependent on intact p38/ERK and PI3K signaling [35, 36]. Therefore, hesperetin and TUDCA act via different mechanisms and the combination of hesperetin and TUDCA may be even more protective than either compound alone. In our study, we determined apoptosis by measuring caspase-3 activity and cleavage of the caspase-3 substrate PARP. So far, in all our studies we always observed a strict correlation between caspase-3 activity and end-points of apoptosis, such as nuclear condensation. Importantly, the reduction of apoptotic cell death by hesperetin was not accompanied by an increase in alternative modes of cell death, such as necrosis as determined by LDH release.

To further dissect the anti-inflammatory and cytoprotective effects of hesperetin, we also evaluated the effect of hesperetin on the cytokine-induced inflammatory response in hepatocytes. We used NF-κB-dependent induction of *Nos2* (iNOS) as a marker of hepatocyte inflammation. Hesperetin significantly inhibited the induction of *Nos2* by cytokines in hepatocytes. Of note, the cytokine mixture alone or in combination with hesperetin did not induce any apoptosis in hepatocytes, again underscoring the independency of the anti-inflammatory and cytoprotective effects of hesperetin. Interestingly, hesperetin induced HO-1 expression, suggesting an opposite effect of hesperetin on iNOS and HO-1 regulation. A reciprocal regulation of iNOS and HO-1 was previously described in intestinal epithelial cells [37]. The induction of HO-1 by hesperetin could, in fact, contribute to the protective effect of hesperetin, since HO-1 is known as a protective and anti-oxidant gene [38, 39].

The *in vitro* effects of hesperetin were confirmed in two mouse models, the T-cell/IFN-γ-mediated model of Con A-induced fulminant hepatitis and the TNFα-mediated model of D-GalN/LPS-induced fulminant hepatitis [21, 40]. In these models, animals were sacrificed 8 h after the challenge, permitting the monitoring of changes in hepatic mRNA expression and apoptosis [41, 42].

Apoptotic signaling within the cell is transduced mainly via the "death receptor" subgroup within the TNF protein superfamily, including TNFα, CD95L (also known as FasL) and the TNF-related apoptosis-inducing ligand (TRAIL) [43]. LPS leads to rapid activation of macrophages and high expression of TNFα in macrophages [44]. Resistance to TNFα cytotoxicity is particularly important in hepatic injury and this resistance is mainly dependent on intact NF-κB-signaling [27]. Therefore, in models of acute TNFα toxicity, NF-κB signaling needs to be suppressed and this is usually accomplished by D-GalN or actinomycin D. In the D-GalN/LPS model of fulminant hepatitis, we observed a very strong inflammatory response, accompanied by extensive liver damage and hepatocyte cell death. Both the inflammatory response and cell injury were strongly inhibited by hesperetin. At least part of the protective effect of hesperetin is due to suppression of inflammation as indicated by reduced leukocyte infiltration and reduced expression of apoptotic cytokines like TNFα and FasL and reduced expression of the endotoxin receptor TLR-4 and the apoptotic ligand FasL. It has previously been shown that Fas/FasL interactions contribute to inflammation in chronic cholestatic liver injury [45]. The reduction in hepatic TLR-4 and FasL expression could be the result of diminished expression of these receptors on inflammatory cells, and/or a reduced infiltration of inflammatory cells due to less cellular injury in the liver. On the other hand, we cannot exclude a direct cytoprotective effect of hesperetin on hepatocytes, as observed in GCDCA-induced hepatocyte apoptosis.

Interestingly, in preliminary experiments using Kunming mice, we also demonstrated that hesperetin protects against D-GalN/LPS-induced fulminant hepatitis when given twice at 200 mg kg^{-1} (1 h and 3.5 h) after challenge, indicating that hesperetin is also therapeutically effective (data not shown).

Although hesperetin protects against apoptotic cell death, the dominant mode of cell death in our models of fulminant hepatitis appears to be necrotic cell death. Although apoptotic cell death was convincingly demonstrated in both models by counting TUNEL-positive cells and measuring caspase-3 activity, the extent of apoptotic cell death is probably limited compared to necrotic cell death in these models. Nevertheless, both indicators of apoptotic cell death were reduced by hesperetin, indicating that hesperetin also prevents apoptotic cell death *in vivo*. Therefore, in our models of fulminant hepatitis, the main protective effect of hesperetin is probably via its action on inflammatory cells resulting in the attenuation of the inflammatory response, less production of inflammatory and apoptotic cytokines and less generation of reactive oxygen species. It should be noted that the contribution of necrotic and apoptotic cell death varies between different liver diseases and that apoptosis is especially relevant in mild to moderate hepatitis like viral hepatitis, (non-)alcoholic steatohepatitis, whereas necrosis is dominant in fulminant hepatitis and acetaminophen intoxication.

Several studies have convincingly demonstrated signaling through the c-Jun N-terminal kinase (JNK) as a critical mechanism of TNFα-induced apoptosis [46], but also in oxidative stress-induced hepatocyte apoptosis [26, 47]. Wang et al. particularly demonstrated that hepatic injury was markedly decreased in mice lacking JNK2 [48] although JNK1 has also been implicated in TNFα-induced hepatitis [44]. Additionally, JNK is essential for development of hepatitis and is required for TNFα expression in hematopoietic cells including resident inflammatory cells in the liver (e.g. Kupffer cells and NKT cells) [49]. Administration of hesperetin attenuated hepatic activation of JNK as determined by Western blot analysis for phosphorylated JNK1/2 (Fig. 6B). It has been demonstrated recently that JNK is also involved in necrotic cell death and that inhibition of JNK attenuates necrotic liver injury [50, 51]. In our study we observe activation (phosphorylation) of JNK and a reduction of JNK activation by hesperetin. Therefore, we conclude that hesperetin can directly inhibit necrotic cell death via inhibition of JNK activation. JNK can be activated by numerous agents, including TNFα, Fas/FasL, reactive oxygen species and bile acids. Therefore, in our models of fulminant hepatitis, with increased expression of TNF and other inflammatory cytokines, as well as generation of reactive oxygen species, it is very likely that JNK is activated via one or more of these ligands.

In contrast to the D-GalN/LPS treatment, the major contributors to liver injury in the Con A model are non-soluble membrane-bound TNFα expressed on macrophages (Kupffer cells) [52] and IFN-γ, expressed in T cells like natural killer T (NKT) cells, which are particularly abundant in the liver. Indeed, high expression of several T-cell cytokines, including IFN-γ, IL-4 and IL-2 has been implicated in Con A-induced hepatitis. Tagawa et al. showed that Con A hepatitis is suppressed in IFN-$\gamma^{-/-}$ mice [53]. In hepatitis B virus-induced hepatitis, NKT cells are recruited to the hepatic parenchyma and contribute to inflammation by releasing cytokines like IL-4 and IFN-γ [54]. In addition to having a direct toxic effect on hepatocytes, IFN-γ may also sensitize liver cells to TNFα-mediated toxicity. Of note, our data demonstrate that hesperetin significantly and dose-dependently reduce serum levels and mRNA expression of IFN-γ and serum level of IL-4. These data support the conclusion that therapeutic administration of hesperetin can also be considered for immune-mediated liver injury and that hesperetin also affects T-cells.

The anti-inflammatory action of hesperetin in Con A-induced fulminant hepatitis is underscored by the reduction of iNOS, a marker for inflammation [55]. This finding paralleled the *in vitro* observation, although in the Con A model we did not distinguish between hepatocyte and Kupffer cell expression of iNOS. It is likely that expression of iNOS in both cell populations is reduced. Preliminary experiments indicate that hesperetin also reduces iNOS expression in the murine macrophage cell line RAW264.7 (data not shown).

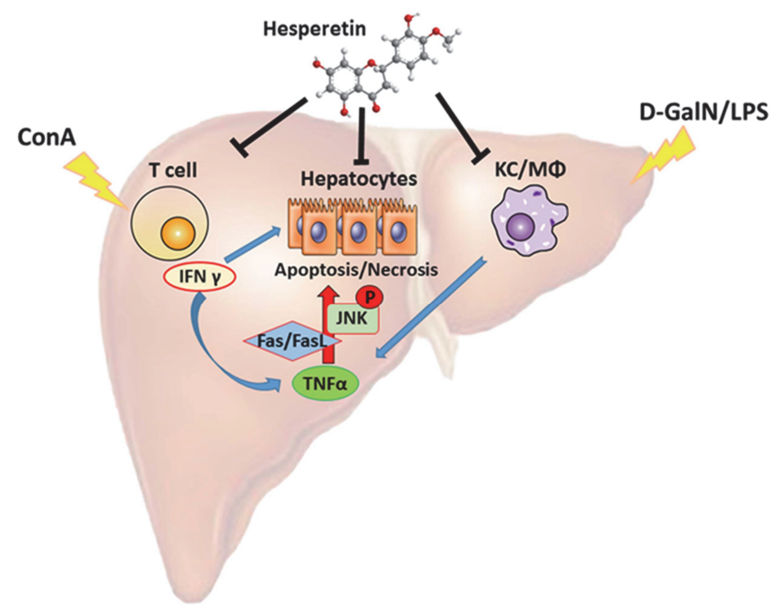

Figure 8. Potential mechanism of hesperetin in fulminant hepatitis. In acute and chronic liver injury, activation and infiltration of inflammatory cells (Kupffer cells, neutrophils) and death of functional hepatocytes occurs. Hesperetin protects hepatocytes from apoptotic cell death induced by TNFα/FasL, activating 'death receptors' and IFN-γ, produced by T cells like Natural Killer T (NKT) cells. Hesperetin has both anti-inflammatory effects on inflammatory cells and protective effects on hepatocytes and these effects are independent. The anti-apoptotic effects of hesperetin are in part due to reduced activation of the pro-apoptotic MAP kinase JNK.

In Fig.8, we propose a potential mechanism of the protective action of hesperetin in fulminant hepatitis. It is important to stress that we propose effects of hesperetin on both the inflammatory and T-cell populations as well as on the hepatocytes. In summary, we demonstrate the therapeutic potential of hesperetin in fulminant hepatitis based on both anti-inflammatory actions of hesperetin on inflammatory cells as well as direct cytoprotective effects of hesperetin on hepatocytes. Therefore, hesperetin has the potential to stop the vicious cycle of inflammation causing cell death and cell death leading to more inflammation as observed in many inflammatory liver diseases. We propose that hesperetin is a promising candidate to be evaluated in clinical studies for the treatment of inflammatory liver diseases, including viral hepatitis, (non-)alcoholic steatohepatitis and fulminant hepatitis.

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Chapter 4

Multifaceted esculetin suppresses hepatic stellate cell activation and CCl_4-induced liver fibrosis in mice

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Manuscript submitted

Abstract

Background: Chronic liver diseases typically lead to liver fibrosis, which may progress to cirrhosis and liver cancer. Liver fibrosis is caused by overactive hepatic myofibroblasts, but is reversible when the disease-causing insult is abrogated. However, no drugs are available that support resolution of hepatic fibrosis. Esculetin (6,7-dihydroxy-coumarin) is a natural compound that inhibits lipoxygenases and has anti-inflammatory, anti-tumor and anti-arrhythmic properties. Inhibition of 5-lipoxygenase in hepatic stellate cells (HSC) suppresses the transdifferentiation to myofibroblasts. Here, we studied the effect of esculetin on hepatic myofibroblasts and CCl_4-induced liver fibrosis in mice.

Methods: Culture-activated primary rat HSC and portal myofibroblasts (PMF) were exposed to esculetin. C57BL/6 mice were treated for 4 weeks with CCl_4 with esculetin co-treatment (5, 10 or 20 μg/g) during the last 2 weeks.

Results: Esculetin inhibited proliferation and expression of activation markers (Col1a1/α-Sma) of *in vitro*-cultured HSC and PMFs. Esculetin dose-dependently reduced CCl_4-induced Sirius-red staining (-64%), Masson trichrome staining (-60%) and hydroxyproline levels (-46%) even below levels observed in 2 week CCl_4-treated mice. Concomitant significant reductions were observed in mRNA and/or protein levels of TGF-β, collagen type 1, fibroblast-specific protein-1, α-Sma and Smad2/3 phosphorylation. Esculetin did not change *Pdgfr-β* and *Pparγ* expression, but did enhance the hepatic ratios of MMP2-9-13/TIMP-1 and GSH/GSSG.

Conclusion: Esculetin halts and partly reverses liver fibrosis under persistent CCl_4 exposure by suppressing profibrotic TGF-β/Smad signaling and myofibroblast proliferation, while enhancing fibrolysis and the anti-oxidant capacity of the liver. Thus, multifaceted esculetin may be a relevant candidate drug to treat liver fibrosis

Introduction

Liver fibrosis is the result of an uncontrolled wound healing response to chronic liver injuries, such as viral and autoimmune hepatitis and (non-) alcoholic fatty liver diseases. Liver fibrosis may progress to cirrhosis in which the liver architecture is irreversibly disturbed and predisposes for liver cancer. Hepatic stellate cells (HSC) play a central role in hepatic fibrogenesis. In the healthy liver, HSC contain most of the body supply of vitamin A stored as retinyl esters in large cytosolic lipid droplets. Upon liver injury, HSC transdifferentiate into hepatic myofibroblasts that loose their vitamin A and become highly proliferative, contractile and produce excessive amounts of extracellular matrix proteins (ECM), including collagens and fibronectins. In addition to HSC, ECM-producing myofibroblasts may also originate from portal myofibroblasts (PMF) and bone marrow-derived mesenchymal stem cells [1, 2]. TGF-β plays a pivotal role in the activation of fibrogenic myofibroblasts, mediated through the Smad pathway. Therapeutic resolution of liver fibrosis may be achieved by suppressing the activation and proliferation of myofibroblasts, reducing the synthesis of excess ECM and/or improving the balance of enzymes that modulate the ECM: matrix metalloproteinases (MMPs) versus tissue inhibitor of metalloproteinases (TIMPs). Despite a multitude of studies focusing on molecular targets in these pathways, no efficient and well-tolerated antifibrotic drugs are available yet. Ideally, an antifibrotic drug would affect different aspects of the process of fibrogenesis, e.g. organ inflammation, myofibroblast activation and/or scar tissue formation.

4

One such product may actually be present in nature. Esculetin (Esc), or 6, 7-dihydroxycoumarin, is present in many plants and medicinal properties have been assigned to stem bark of *Fraxinus rhynchophylla* [3] and the herbs *Cichorium intybus* (chicory) [4-6] and *Artemisia capillaris* (Compositae) [7, 8], which contain high levels of this compound. Esculetin possesses multiple pharmacological activities, including analgesic, anti-inflammatory [8, 9], anti-tumor [10, 11], anti-arrhythmic [12], antisteroidogenic [13, 14] properties. Mechanistically, it has been shown to suppress cell proliferation, particularly of cancer cells [15, 16]. Furthermore, it acts as an anti-oxidant and inhibits apoptosis, e.g. of hepatocytes exposed to CCl_4 [17, 18]. Esculetin is a non-competitive inhibitor of 5-lipoxygenase and 12/15-lipoxygenase, enzymes that produce leukotrienes and lipoxins. Both enzymes have independently been show to promote fibrosis in various organs, including lung [19] and heart [20-22] Hepatic lipoxygenase expression was assumed to be restricted to Kupffer cells [23, 24], but we [25] and others [26] have recently found that HSC also express 5-LO and that 5-LO is induced upon transactivation of myofibroblasts *in vitro*. Inhibition of 5-LO, both transcriptionally and pharmacologically, suppressed HSC proliferation and reduced expression of HSC activation markers collagen 1a1 and α-Sma. *5-Alox* knockout mice show resistance to hepatic inflammation and develop markedly reduced fibrosis when exposed to hepatotoxic agents like CCl_4 [27]. Esculetin effectively prevents acute liver damage in mice [17] and rats [18, 28, 29] caused by a single dose of CCl_4. In chronic liver injury models, esculetin is typically given simultaneously with the liver damage-causing agent/condition without or with a short pre-treatment with esculetin. Despite its potent hepatocyte-protective effect, its true value in treatment of liver fibrosis with hepatic myofibroblasts as the main target remains elusive. Here, we studied the direct effect of esculetin on primary rat HSC and its potential as an antifibrotic drug after CCl_4-induced liver injury in mice with established liver fibrosis.

Materials and methods

Animals and CCl_4-induced liver fibrosis

Male C57BL/6 mice (Vital River Laboratories, Beijing, China) were housed in the SPF animal facility of Shantou University Medical College under standard 12hr light/12hr dark cycle and fed standard rodent chow and water ad libitum. After 7 days adjustment, mice were randomly divided into eight treatment groups (CCl_4 groups n=10; control groups n=6). Hepatic fibrosis was induced as described before [30] by twice-weekly injections of CCl_4 (i.p.; 0.4 µl/g BW) diluted 1:3 in olive oil CP (Aladdin Chemistry Co. Ltd, Shanghai, China) for 4 weeks (total nine injections). Esculetin treatment (Alfa Aesar, MA, USA; i.p. 5µg/g, 10µg/g or 20µg/g BW in PBS) was started at week 3 (together with 5th CCl_4 injection), given once a day for 16 days in total, with continued CCl_4 treatment. Mice injected with CCl_4 for 2 weeks and 4 weeks were included for comparison. The latter group received daily mock injections with PBS as control for esculetin treatment. Control mice received olive oil-injections for 2 or 4 weeks. Additionally, one group received esculetin alone at the highest dose (20µg/g) for 16 days. Mice were sacrificed 48 h after the last CCl_4 injection. Body weight was determined before every CCl_4 injection. Blood samples were collected at sacrifice for biochemical analyses. The left lateral liver lobe was prepared for histological analyses. Remaining liver tissue was snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein isolation.

Isolation and culture of primary rat hepatic stellate cells and portal myofibroblasts

Specified pathogen-free male Wistar rats (Harlan, Zeist, the Netherlands) were housed under standard laboratory conditions with free access to standard laboratory chow and water. Experiments were approved by the local committee for care and use of laboratory animals of the University of Groningen, the Netherlands.

Hepatic stellate cells (HSC) were isolated from 500-600g male rats as described previously [31]. Cell purity was at least 90% after isolation and 100% after 1 day of culturing. HSC were culture-activated for 7 days. HSC were passaged by trypsinization. Cells at passage 1 or 2 were used for experiments.

Portal myofibroblasts (PMF) were isolated from residues of portal tree of 220-250g male rat as described earlier [32]. Freshly-isolated PMF were passed after 5 days and passages 2-4 were used for experiments HSC and PMF were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with Glutamax (Invitrogen, Breda, the Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mmol/L sodium-pyruvate (Invitrogen), 1×MEM non-essential amino acids (Invitrogen), 50 μg/mL gentamicin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 μg/ml streptomycin (Lonza), 250 ng/mL fungizone (Lonza) in a humidified atmosphere containing 5% CO_2 at 37°C. Culture-activated HSC and PMF were treated with esculetin (50-100 μmol/L) in 20% serum-containing medium for the indicated time points.

RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from liver tissue using RNAiso Plus (Total RNA extraction reagent TaKaRa, Japan) and from HSCs and PMFs using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. Purity and concentration were analyzed by Nanodrop 2000 (Thermo Scientific). 1 μg of total RNA was reverse transcribed using PrimeScript ™ RT reagent Kit with gDNA Eraser (Perfect Real Time TaKaRa, Japan; for liver tissue) or using random nanomers (Sigma-Aldrich; for HSC and PMF). Quantitative reverse-transcription polymerase chain reaction assays were performed on the Eco Real-Time PCR System (Illumina USA) using SYBR Green reagent (TaKaRa, Japan) or ABI PRISM 7700 (PE Applied Biosystems) using taqman probes. Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (Ct value) were normalized to the endogenous control (*Gapdh* for liver tissue; *36B4* for HSC and PMF) and correlated inversely with initial mRNA levels. Relative quantification was performed using the ddCt method. Real time PCR primers and probes are given in Supplementary Table S1.

Biochemical analyses

ALT, AST, and LDH were quantified using the Bio-sinew (Chengdu, China) and Leadman (Beijing, China) kits and the IFCC recommend method on an Automatic Chemistry Analyzer (Accute TBA-40FR, Toshiba medical systems corporation, Japan). Serum levels of mouse TIMP-1 were measured by quantikine ELISA (R&D systems, USA). Tissue hydroxyproline content was determined using the Chloramine-T Hydroxyproline Assay Kit (Nanjing Jiancheng Biotechnology Institute Co., Ltd., Nanjing, China.) according to the manufacturer's protocol. Tissue glutathione levels were determined using the GSH/GSSG assay kit (Beyotime, Jiangsu, China) according to the manufacturer's protocol.

SDS-PAGE and Western blot analysis

Liver samples were homogenized in RIPA lysis buffer (P0013B; Beyotime). Equal amounts of proteins (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose filter membranes. Following blocking, membranes were probed with primary antibodies: mouse anti α-SMA monoclonal antibody 1:2,000 (Sigma); mouse phospho-Smad2(Ser465/467)/Smad3(Ser 423/425) (D27F4), rabbit monoclonal antibody 1:1,000 (Cell Signaling Technology, USA), anti-TGF-β antibody ab66043 1:1,000 (Abcam, Cambridge, UK), mouse anti-GAPDH monoclonal antibody 1:3,000 (ZSGB-BIO, Beijing), followed by HRP-conjugated secondary mouse antibody 1:60,000 or secondary rabbit antibody 1:80,000 (ZSGB-BIO, Beijing) at room temperature for 1 h. Target proteins were visualized using an enhanced chemiluminescence SuperSignal West Dura detection system (Thermo scientific, IL, USA). Signals were quantified by Quantity One (Bio-Rad).

Gelatin zymography

Gelatinase substrate gel electrophoresis was performed using 30% polyacrylamide gels containing 1% gelatin. Samples were prepared in 2x loading buffer consisting of 1 mol/L Tris, pH 6.8, 10% SDS, 20% glycerol, 0.2% bromophenol blue. 50 μg protein was loaded per sample. After electrophoresis, gels were incubated twice in renaturing solution (2.5% Triton X-100) for 30 minutes at room temperature and then for 24 hours at 37°C in a developing buffer containing 50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 1% Triton X-100. Gels were stained with Coomassie Brilliant Blue R-250. MMP-2 and MMP-9 gelatinolytic activity was quantified by Quantity One (Bio-Rad).

Cell Proliferation Assay

Activated HSC or PMF were seeded in a 96-well plate and grown for 3 days in the absence or presence of esculetin. Proliferation was assessed at day 3 allowing BrdU incorporation for 3 h using cell proliferation ELISA kit (Roche, Almere, the Netherlands) according to the manufacturer's instructions and measured spectroscopically (The Synergy™HT, BioTek Instruments, Inc.).

Histology and immunohistochemical studies

Liver samples were fixed in 4% paraformaldehyde and embedded in paraffin. 4-μm paraffin sections were processed following routine methods. Masson trichrome and Sirius-red staining (Beijing Leagene Biotechnology Co.,Ltd.) were performed according to manufacturer's instructions. Deparaffinized liver sections were immunostained for α-SMA (Sigma; dilution 1:200) using a two-step immunohistochemistry kit (Super Vision, Boster, Wuhan, China). Staining was determined in at least 10 separate fields and quantified using a computer-aided image analysis system IPP6.0.

Immunofluorescence microscopy

HSC and PMF were cultured on glass coverslips and fixed in 4% paraformaldehyde/PBS. Cells were permeabilized by 1% Triton X-100 for 5 min.

Nonspecific antibody binding sites were blocked in 0.5% BSA/PBS for 30 min. Next, cells were incubated with primary antibodies α-SMA (Sigma Aldrich, 1:500) and collagen 1α1 (Southern Biotech, 1:30) in 0.5% BSA/PBS for 1-2 h at room temperature. Secondary antibodies were Alexa fluorophores (Molecular Probes, 1:500). Coverslips were mounted in fluorescence mounting medium S3023 containing DAPI for nuclear staining (DAKO). Staining was visualized using a Zeiss 410 inverted laser scan microscope.

Statistical analysis

All data are presented as mean ± standard deviation. Significance of differences between groups was tested by one-way ANOVA and t-test. Calculations were made using the software of GraphPad Prism 5. Results were considered statistically different when the p value <0.05.

Results

Esculetin inhibits proliferation of primary rat HSCs and PMFs

First, we analyzed whether esculetin directly affects proliferation and/or activation of the 2 main sources of hepatic myofibroblasts, portal myofibroblasts (PMF) and hepatic stellate cells (HSC) (Fig. 1). Culture-activated primary rat PMF and HSC were treated for 3 days with or without esculetin (50 or 100 μmol/L). Both BrdU incorporation (Fig. 1A) and PCNA protein levels (Fig. 1B) were sharply decreased in PMF and HSC after esculetin treatment, suggestive of a potent suppression of cell proliferation. Messenger RNA (Fig. 1C) and protein levels (Fig. 1D) of collagen1a1 and α-Sma were slightly reduced after treatment with 50 μmol/L esculetin, but a clear reduction of these myofibroblast activation markers was observed at higher concentrations (100 μmol/L) of esculetin (Fig. 1D). No significant induction of PMF or HSC cell death was observed at these conditions as indicated by the absence of cellular LDH release during the course of the treatment (Supplementary Fig. S1).

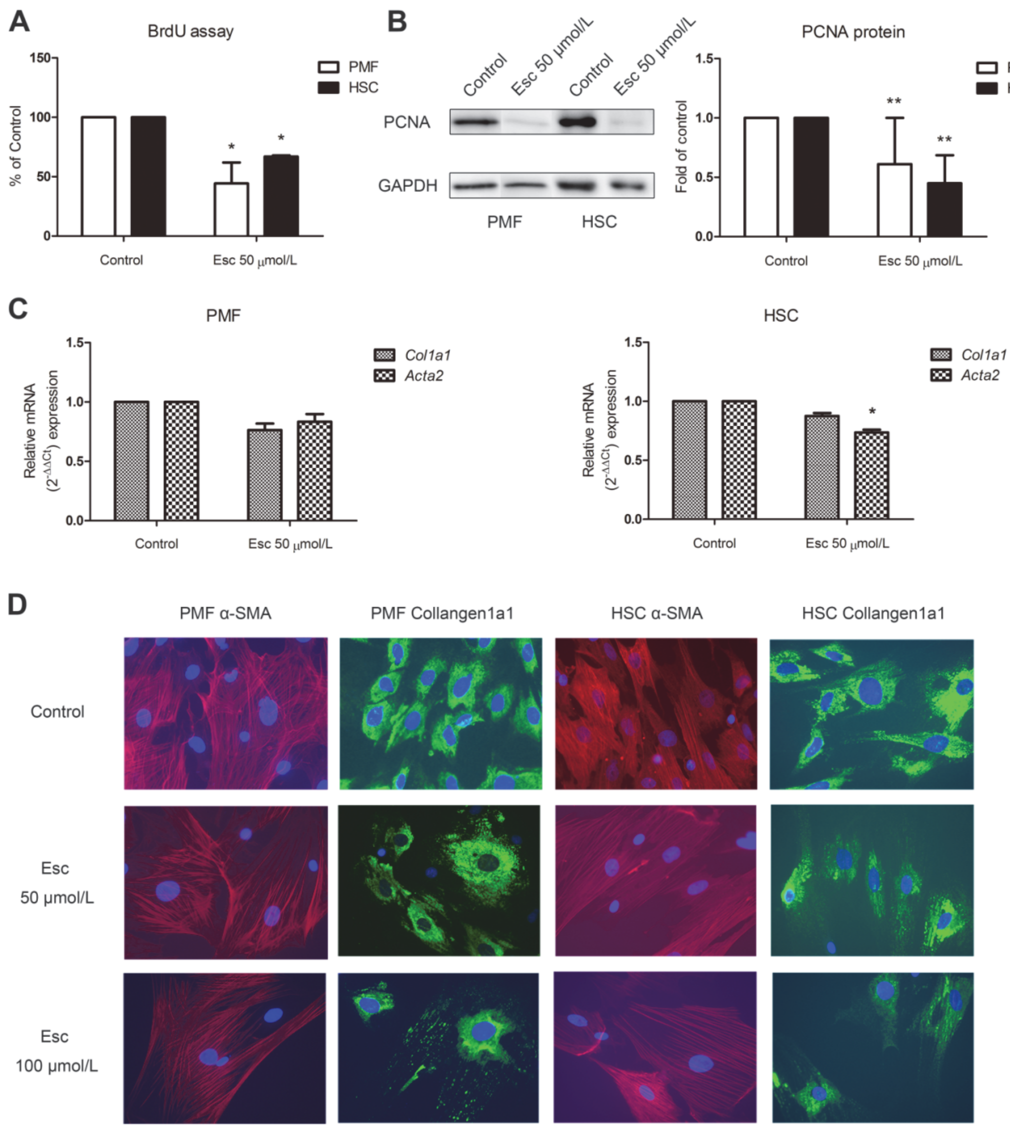

Figure 1. Esculetin inhibits proliferation and activation of primary rat HSC and PMF. Primary HSC and PMF were cultured for 3 days in the absence or presence of esculetin. BrdU incorporation was quantified on day 3 (A). PCNA protein levels (B) and *Col1a1* and *Acta2* mRNA levels (C) were determined by Western blotting and Q-PCR, respectively. (D) Immunofluorescent microscopy of intracellular collagen (green) and α-Sma (red) in PMF (left panels) and HSC (right panels) in the absence (top panels) and presence of 50 or 100 μmol/L esculetin (Esc) (original magnification 200×). *p <0.05, **p <0.01 compared to control. Experiments were performed from at least 3 different HSCs and PMFs isolations.

Esculetin halts progression and partly reverses liver fibrosis in CCl_4 treated mice

Esculetin has been shown to protect hepatocytes against acute toxicity by a single high dose of CCl_4. To determine its efficacy as an antifibrotic drug, we subjected mice to the chronic model of CCl_4-induced fibrosis, where mice were given CCl_4 injections twice a week for 4 weeks and esculetin treatment was started from week 3 on a daily basis until sacrifice at the end of week 4. Body weight characteristics were comparable for all CCl_4-treated groups, showing an initial drop after the first CCl_4 injection after which body weight gain is observed during the consecutive CCl_4 treatments (Fig. 2A). Liver weight at sacrifice was similar for all experimental groups and comparable to the untreated controls (Fig. 2B). Serum transaminases (ALT, AST) were strongly elevated after 2 weeks CCl_4 treatment (Fig. 2C). After 4 weeks CCl_4, AST and LDH were not significantly elevated anymore, while ALT levels were 40% reduced compared to 2 week CCl_4-treated mice, but still significantly enhanced above normal levels. Albumin and total bilirubin levels were comparable in all groups and not different from untreated controls (Supplementary Fig. S2). Sirius-red (Fig. 2D) and Masson trichrome (Fig. 2E) staining progressively increased in livers after 2 and 4 week CCl_4 treatment, with evident perilobular fibrosis and marked bridging fibrosis after 4 week CCl_4 treatment. Hepatic hydroxyproline levels were significantly enhanced in 2-week and 4-week CCl_4 treated mice compared to controls (Fig. 2F).

Esculetin dose-dependently reduced serum ALT levels in CCl_4 treated mice, but levels remained increased compared to untreated controls (Fig. 2C). AST and LDH levels were not elevated after 4-week CCl_4 treatment and esculetin did not change this. Sirius-red and Masson trichrome staining revealed that esculetin dose-dependently reduced hepatic collagen deposition in CCl_4-treated mice (Fig. 2D and E). The Sirius red-positive area dropped from 3.99% in 4 week CCl_4-treated mice to 1.44% in animals receiving the highest dose of esculetin, which was similar to the level after 2 weeks CCl_4 treatment (Fig. 2D). Even more pronounced, esculetin reduced the Masson trichrome-positive area from 8.45% to 3.39% after 4 weeks of CCl_4 treatment, which is significantly lower compared to mice treated for 2 weeks with CCl_4 (6.17%) (Fig. 2E). Similarly, esculetin dose-dependently reduced hepatic hydroxyproline below levels in livers from mice treated for 2 weeks CCl_4, marking the start of esculetin treatment (Fig. 2F). In line with the histological observations, hepatic mRNA levels of *collagen 1a1*, *Acta2* and fibroblast-specific protein (*Fsp1*) were dose-dependently reduced by esculetin (Fig. 3A) and accompanied with a reduction in α-Sma protein (Fig. 3B). Strong periportal staining was observed for α-Sma in livers of 4 week CCl4-treated mice, which was almost completely absent when co-treated with the highest dose of esculetin (Fig. 3C). Moreover, parenchymal staining of α-Sma was observed in the initial stages of fibrosis after 2 week CCl4-treatment, which was not detected in mice subsequently treated for 2 weeks with CCl4 together with 20 µg esculetin/g body weight (Fig. 3D).

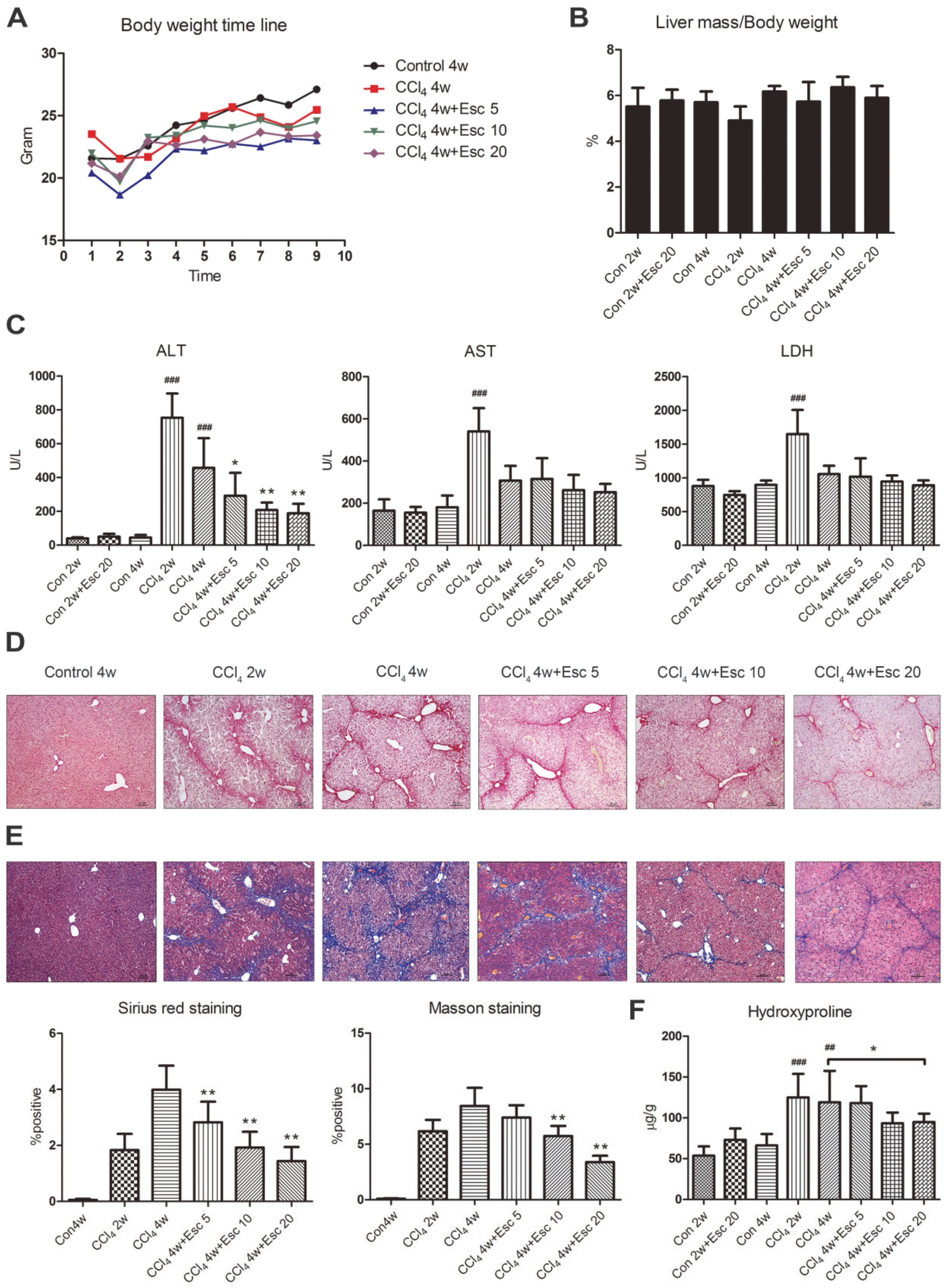

Figure 2. Esculetin halts progression and partly reverses liver fibrosis under persistent CCl_4 administration in mice. (A) Body weight development during CCl_4 treatment ("Time" indicates CCl_4 injections). (B) Liver mass as percentage of body weight at sacrifice. (C) Serum liver damage makers (ALT, AST and LDH) at sacrifice. Collagen deposition as determined by Sirius-red staining (D) and Masson trichrome staining (E) (original magnification 200×) including quantification of staining by densitometry. (F) Hepatic hydroxyproline content (μg/g). *p <0.05, **p <0.01, compared to CCl_4 4w. ##p <0.01, ###p <0.001 compared to control 4w.

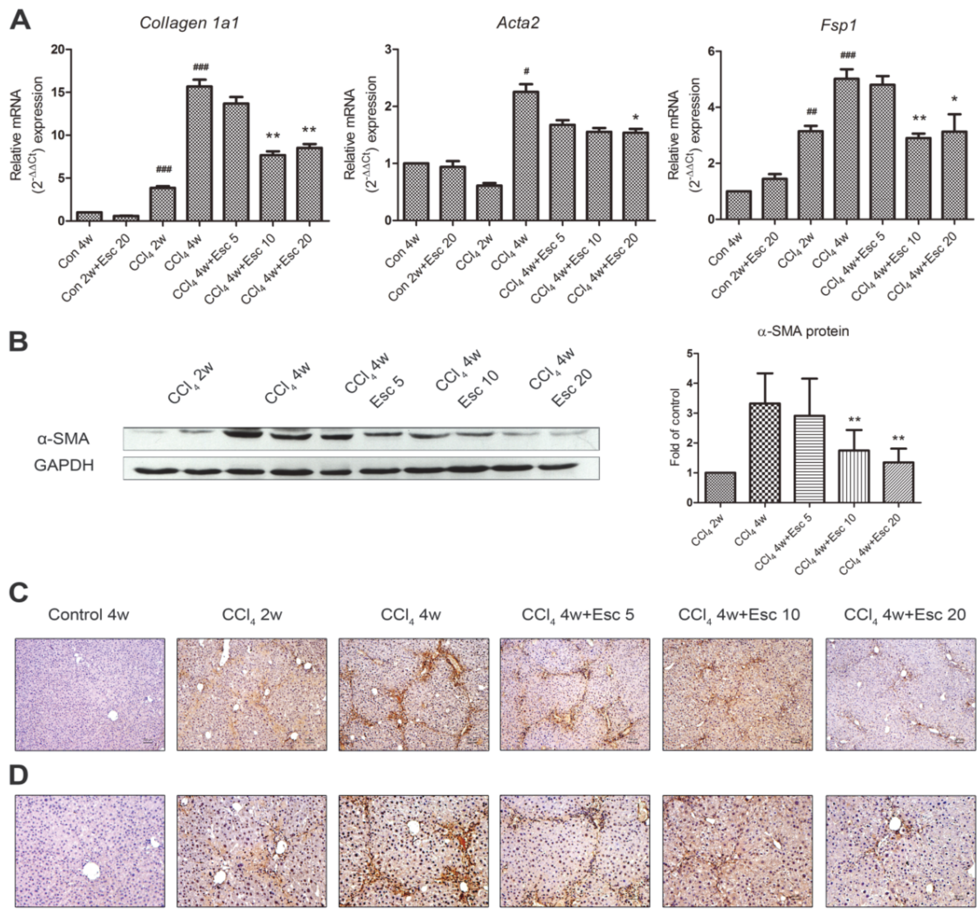

Figure 3. Esculetin suppresses CCl_4-induced mRNA and protein expression of hepatic myofibroblast activation markers. Liver homogenates analyzed for (A) mRNA levels of *Collagen 1a1*, *Acta2* and *Fsp1* by Q-PCR and (B) α-SMA protein expression by Western blotting. GAPDH was used as loading control. Protein bands were quantified by densitometry and corrected for GAPDH. (C) Immunohistochemical staining for α-SMA (original magnification 200×). (D) shows a zoom of (C) providing details of the cellular distribution of α-Sma staining in the parenchyma. *$p < 0.05$, **$p < 0.01$ compared to CCl_4 4w. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to control 4w.

Esculetin differentially affects lipoxygenase mRNA expression

Esculetin is a non-competitive inhibitor of 5-lipoxygenase (5-LO) and 12/15-lipoxygenase (12/15-LO). We did not observe compensatory expression of either of the enzymes in the esculetin-treated mice. Both *Alox5* (16-fold) and *Alox15*

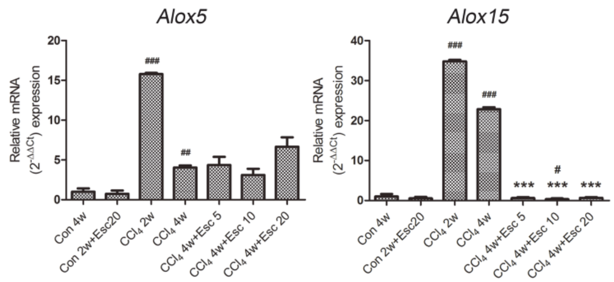

Figure 4. Differential effect of esculetin on CCl_4-induced lipoxygenase expression. *Alox5* and *Alox15* mRNA levels were strongly increased after 2 week CCl_4 treatment after which both decreased at 4 week CCl_4 treatment, but remain elevated compared to untreated control. Esculetin did not affect *Alox5* mRNA levels, but fully suppressed *Alox15* expression. ***p <0.001 compared to CCl_4 4w. #p <0.05, ##p <0.01, ###p <0.001 compared to control 4w.

(35-fold) mRNA levels were strongly increased after 2 weeks CCl_4 treatment (Fig. 4), after which they decreased at 4 weeks CCl_4 treatment to 4-fold and 23-fold enhanced levels compared to untreated controls, respectively. Esculetin treatment during continued CCl_4 administration did not change *Alox5,* mRNA levels (Fig. 4). In sharp contrast, esculetin fully suppressed *Alox15* mRNA levels in some conditions (10 µg Esc/g body weight) even below levels observed in untreated animals (Fig. 4).

Esculetin suppresses Tgf-β/Smad-signaling

As expected, Tgf-β expression was progressively induced after 2- and 4- week CCl_4 treatment (Fig. 5A). Esculetin (at doses of 10 and 20 µg/g body weight) inhibited Tgf-β expression both at mRNA and protein level (Fig. 5A,B), which was accompanied by reduced phosphorylation of the downstream effector proteins Smad2/3 (Fig. 5B). In contrast, expression of key factors of other pathways that may contribute to CCl_4-induced liver fibrosis, including *Pdgfr-β* and *Pparγ*, were not changed by esculetin (Fig. 5A).

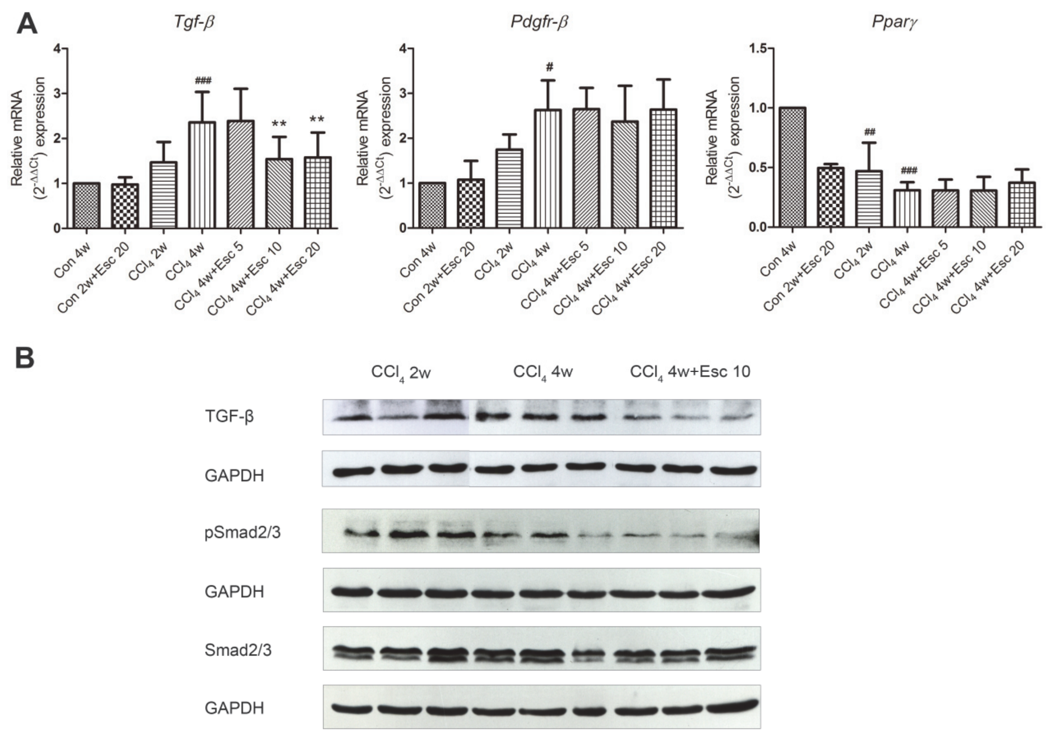

Figure 5. Esculetin suppresses CCl_4-induced TGF-β/Smad signaling. CCl_4 induced mRNA levels of *Tgf-β* and *Pdgfr-β*, while reducing *Pparγ* (A) the typical signature of the development of fibrosis. Esculetin only affected *Tgf-β* transcript levels, which was accompanied by reduced hepatic TGF-β protein levels and Smad2/3 phosphorylation, as determined by Western blotting (B). **p <0.01 compared to CCl_4 4w. #p <0.05, ##p <0.01, ###p <0.001 compared to control 4w.

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Esculetin affects Timp-1 and Mmp expression towards fibrosis resolution.

CCl_4 progressively induced mRNA levels of *Mmp2* and *Mmp9*, while *Mmp13* expression peaked at 2 weeks (29-fold), but remained significantly enhanced (14-fold) compared to controls after 4 weeks CCl_4 treatment (Fig. 6A). Esculetin only reduced *Mmp9* mRNA expression (-52%), which was accompanied by reduced MMP9 activity (-25%; Fig. 6B). Most pronounced was the effect of esculetin on CCl_4-induced *Timp1* expression (+3.2-fold; Fig. 6C). *Timp1* mRNA levels were fully suppressed to control levels, which was accompanied by strongly reduced TIMP-1 protein levels in serum.

Esculetin improves the hepatic GSH/GSSG balance in CCl_4 treated mice

Reduced glutathione (GSH) levels in the liver were decreased after 4-week CCl_4 treatment, which was accompanied by enhanced oxidized glutathione (GSSG) levels (Fig. 7). This leads to a strongly decreased GSH/GSSG ratio, which was significantly enhanced in all esculetin-treated groups (Fig. 7), implying that esculetin significantly improves the hepatic anti-oxidant status under hepatotoxic CCl_4 treatment.

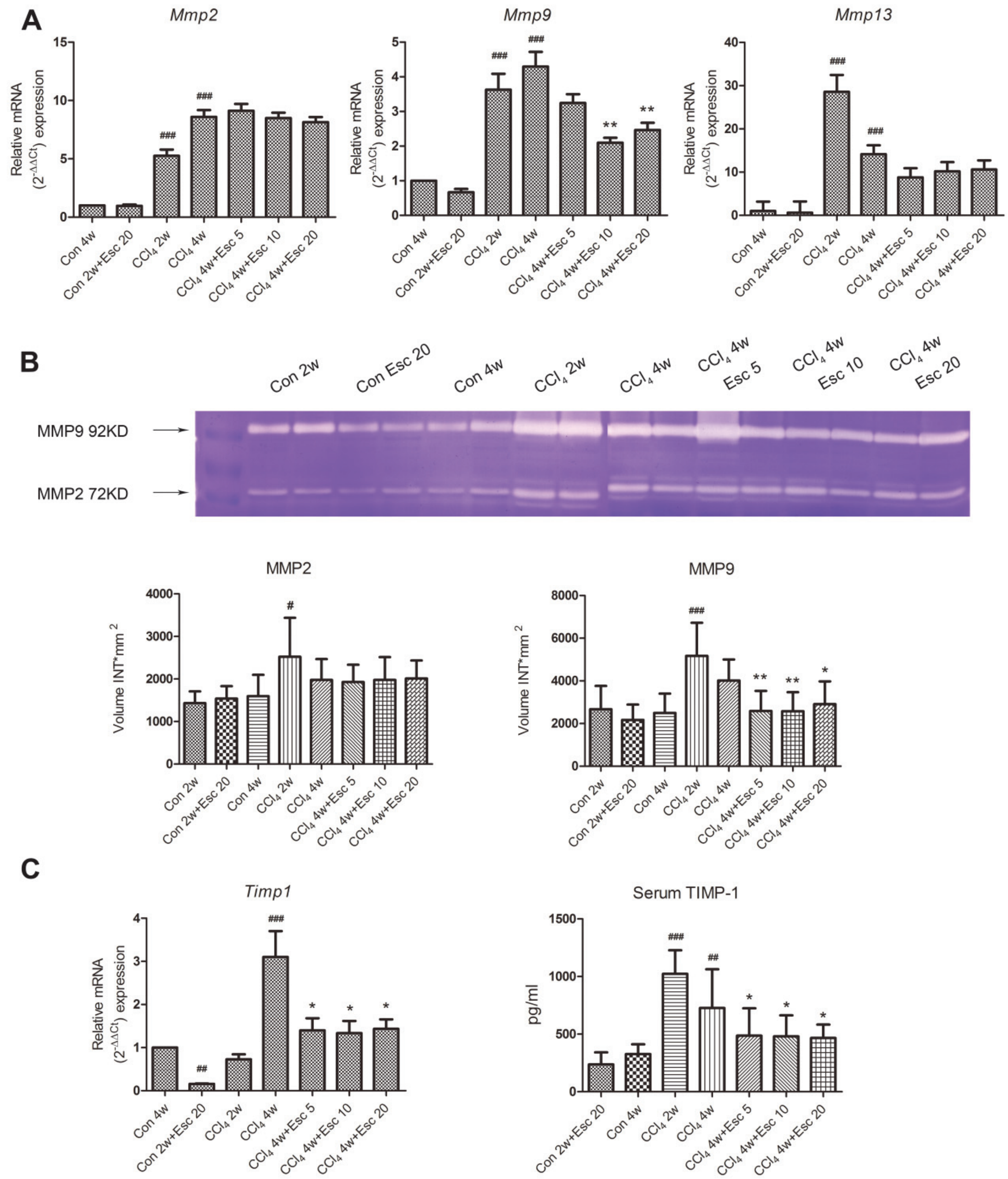

Figure 6. Esculetin promotes fibrolysis by increasing the MMP2-9-13/TIMP-1 ratio. (A) CCl_4 strongly enhanced mRNA levels of *Mmp2*, *Mmp9* and *Mmp13*. Esculetin did not change (*Mmp2* and *Mmp13*) or only moderately reduced (*Mmp9*) metalloproteinase expression. MMP2 and MMP9 activity were confirmed by zymography and quantified by densitometry (B). (C) CCl_4 strongly induced hepatic *Timp-1* mRNA levels (left panel), which was fully suppressed by esculetin. Serum TIMP-1 levels (right panel) mirrored hepatic *Timp-1* transcript levels. *p <0.05, **p <0.01 compared to CCl_4 4w. #p <0.05, ##p <0.01, ###p <0.001 compared to control 4w.

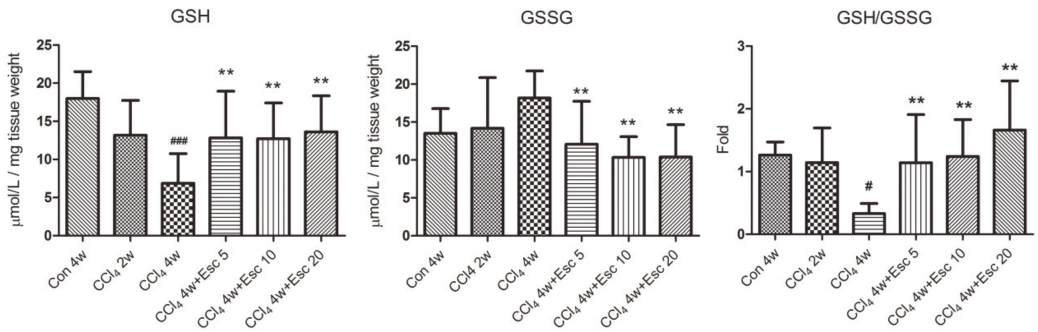

Figure 7. Esculetin improves the hepatic GSH/GSSG balance in CCl_4 treated mice. Reduced (GSH) and oxidized (GSSG) glutathione levels were quantified in total liver homogenates and used to calculate the GSH/GSSG ratio. CCl_4 induced a significant reduction in GSH/GSSG ratio which was ameliorated by esculetin treatment. **p <0.01 compared to CCl_4 4w. #p <0.05, ###p <0.001 compared to control 4w.

Discussion

In this study, we show that esculetin directly suppresses proliferation and activation of hepatic myofibroblasts *in vitro*. Esculetin halts and in part reverses liver fibrosis (e.g. hydroxyproline content, Masson trichrome staining) during persistent CCl_4-induced liver injury. The TGF-β/Smad signaling pathway was suppressed by esculetin, while PPARγ and PDGFR-β expression was not affected. Expression of metalloproteases (MMP2, 9 and 13) were reduced by esculetin, but remained elevated compared to untreated controls. In contrast, CCl_4-induced TIMP-1 expression was fully suppressed by esculetin. In addition, esculetin rescued the CCl_4-associated reduction in hepatic GSH/GSSG ratio. Thus, esculetin suppresses profibrotic signaling, improves fibrolysis and maintains an anti-oxidant status during CCl_4-induced liver toxicity.

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Esculetin is a natural product and is a main constituent of medicinal plant extracts. A plethora of therapeutic properties have been assigned to esculetin, including analgesic, anti-inflammatory [8, 9], anti-tumor [10, 11], anti-arrhythmic [12], antisteroidogenic [13, 14] properties. With respect to hepatoprotective activities, esculetin has so far only been studied in models of acute liver toxicity, such as t-butyl hydroperoxide [33] and high single-dose CCl_4 [29]. Esculetin is a non-competitive inhibitor of 5-lipoxygenase and 12/15-lipoxygenase. These enzymes are essential for leukotriene and lipoxin synthesis that are potent inflammatory signaling molecules. We and others have shown that rat HSC express *Alox5* [26] and is under inhibitory control of the retinoid-related orphan receptor-alpha (RORα). Both RORα agonists (melatonin, SR1078) and a 5-LO antagonist (AA861) suppress HSC proliferation and activation [25]. In line, several recent studies have shown that melatonin potently suppresses liver fibrosis in the chronic CCl_4-model in mice and rats [34-37]. Part of these effects may be exerted by the suppression of 5-LO. Long-term drug treatment with melatonin to treat liver fibrosis may, however, not be an ideal approach as this pineal gland hormone controls circadian rhythm and induces sleep [38-40]. Thus, we reasoned that esculetin, as a direct inhibitor of 5-LO and a component of save medicinal plant extracts may be an attractive alternative.

Hepatic expression of *Alox5* and *Alox15* was strongly induce by CCl_4 treatment with highest levels after 2 weeks, at which point the esculetin treatment started. Esculetin treatment did not change hepatic *Alox5* levels, reminiscent of its effect on 5-LO enzyme activity and not on transcriptional regulation, while *Alox15* expression returned to levels observed in untreated animals. Remarkably, we were unable to detect *Alox15* expression in freshly-isolated (quiescent) or culture-activated rat HSC (data not shown), suggesting that these effects occur in non-HSC liver cells, most likely including Kupffer cells [41]. It remains to be determined whether esculetin directly affects transcription of the *Alox15* gene or whether this is a result of feedback mechanisms associated with reduced inflammation and/or fibrosis.

Esculetin has been shown to induce expression PPARγ and suppress expression of PDGF [42], TGF-β [43, 44],various MMPs and TIMP-1 [45, 46], all factors that are involved in fibrogenesis and/or fibrolysis.

In CCl_4 treated mice, esculetin did not change *Pparγ* and *Pdgfr-β* expression. Instead, significant suppression of TGF-β expression and downstream Smad2/3 phosphorylation was observed, suggesting that this profibrotic pathway was most sensitive to esculetin. Most pronounced, however, was the strong suppression of CCl_4-induced TIMP-1. TIMP-1 is a generic inhibitor of MMPs and thereby prevents fibrosis resolution. CCl_4-induced expression of *Mmp2* and *Mmp13* were not changed by esculetin, while *Mmp9* was only moderately reduced. Thus, the esculetin-increased MMP/TIMP-1 balance favors fibrolysis.

Esculetin strongly suppressed proliferation, as well as collagen1a1 and αSma expression, of *in vitro* cultured HSC and PMF. These results are highly similar as effects observed with melatonin, SR1078 (RORα antagonist) and AA861 (5-LO antagonist), and suggest that these all act (in part) through inhibition of 5-LO. The anti-proliferative properties of esculetin are well-documented, especially in relation to cancer cells. Though this has not been specifically studied for the various forms of liver cancer (cells) yet, this feature may also be beneficial for preventing progression of cirrhosis to liver cancer.

Finally, we also found that esculetin prevents the CCl_4-induced reduction of the GSH/GSSG balance, thereby preserving the anti-oxidant capacity in the liver. Most of the glutathione in the liver resides in hepatocytes, suggesting that esculetin also has a cytoprotective effect on the major liver cell type, as has been documented before. It is important to note that the esculetin did not fully prevent CCl_4-induced hepatocyte damage as indicated by elevated transaminases in the absence and presence of esculetin. Thus, the antifibrotic effect of esculetin cannot be explained solely by protection of the hepatocytes.

Recent reports revealed very similar effects of melatonin and *Fraxinus rhynchophylla* extracts on CCl_4-induced liver fibrosis. Remarkably, the concentrations of esculetin present in the *Fraxinus rhynchophylla* extracts (given at 0.1, 0.5 and 1.0 mg/g body weight with 33.54 mg esculetin/mg *Fraxinus rhynchophylla* extract) were in the same range as our study (5, 10 and 20 µg esculetin/g body weight). This suggests that esculetin may be the most prominent antifibrotic factor in this plant extract.

Taken together, this study reveals multiple antifibrotic properties of esculetin, including suppression of hepatic stellate cell activation, profibrotic TGF-β/Smad signaling and induction of fibrolysis by enhancing the MMP/TIMP-1 balance. Moreover, the anti-oxidant capacity of the liver is improved by increasing the GSH/GSSG ratio.

These results warrant follow-up studies with the long term application of esculetin before and at various stages of established fibrosis to further establish it preventive and therapeutic capacity in the treatment of liver fibrosis. Moreover, experiments to test the anti-fibrotic potential on human liver, e.g. using precision-cut human liver slices [47], are urgently needed.

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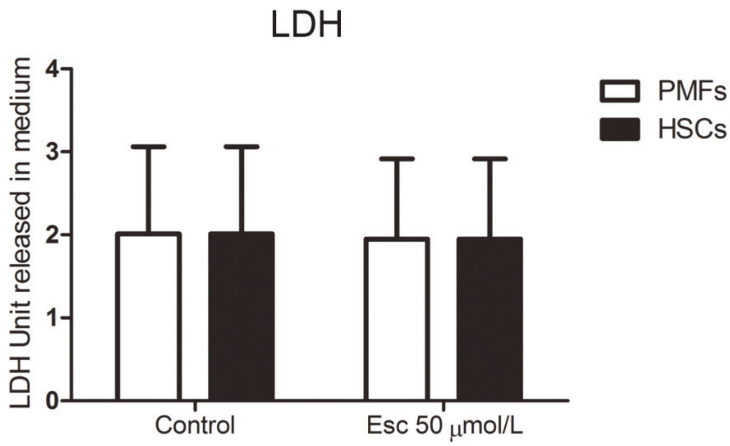
Supplementary Material

Supplementary Table S1. Real time PCR primers used in this study

Gene	Forward primer	Reverse primer	Probe	Locus
r36B4	GCTTCATTGTGGGAG CAGACA	CATGGTGTTCTTGCC CATCAG	TCCAAGCAGATGCA GCAGATCCGC	NM_022402
rActa2	GCCAGTCGCCATCA GGAAC	CACACCAGAGCTGT GCTGTCTT	CTTCACACATAGCTG GAGCAGCTT-CTCGA	NM_031004
rCol1a1	TGGTGAACGTGGTGT ACAAGGT	CAGTATCACCCTTGG CACCAT	TCCTGCTGGTCCCCG AGGAAACA	NM_007742
mActa2	GGAGAAGCCCAGCC AGTCGC	AGCCGGCCTTACAG AGCCCA		NM_007392.3
mCol1a1	ACTTCAGCTTCCTGC CTCAG	TGACTCAGGCTCTT GAGGGT		NM_007742.3
mFSP1	GGAGCTGCCTAGCTT CCTG	GCTGTCCAAGTTGCT CATCA		NM_011311.2
mMmp2	GCCCCGAGACCGCT ATGTCCACT	GCCCCACTTCCGGTC ATCATCGTA		NM_008610.2
mMmp9	GCGCCACCACAGCC AACTATG	TGGATGCCGTCTATG TCGTCTTTA		NM_013599.3
mMmp13	CCTTCTGGTCTTCTG GCACAC	GGCTGGGTCACACTT CTCTGG		NM_008607.2
mTimp1	ACTCGGACCTGGTCA TAAGGGC	TTCCGTGGCAGGCAA GCAAAGT		NM_011593.2
mPdgfr-β	TCAACGACTCACCA GTGCTC	TTCAGAGGCAGGTA GGTGCT		NM_008809.2
mTgf-β	TTGCTTCAGCTCCAC AGAGA	TGGTTGTAGAGGGCA AGGAC		NM_011577.1
mPparγ	ATTCTGGCCCACCAA CTTCGG	TGGAAGCCTGATGCT TTATCCCCA		NM_011146.3
mAlox5	CGGCTTCCCTTTGAG TATTGATGC	CAGGAACTGGTAGC CAAACATGAG		NM_009662.2
mAlox15	ATGGTGCTGAAGCG GTCTAC	ATCCGCTTCAAACAG AGTGC		NM_009660.3
mGAPDH	GCACAGTCAAGGCC GAGAAT	GCCTTCTCCATGGTG GTGAA		NM_008084.2



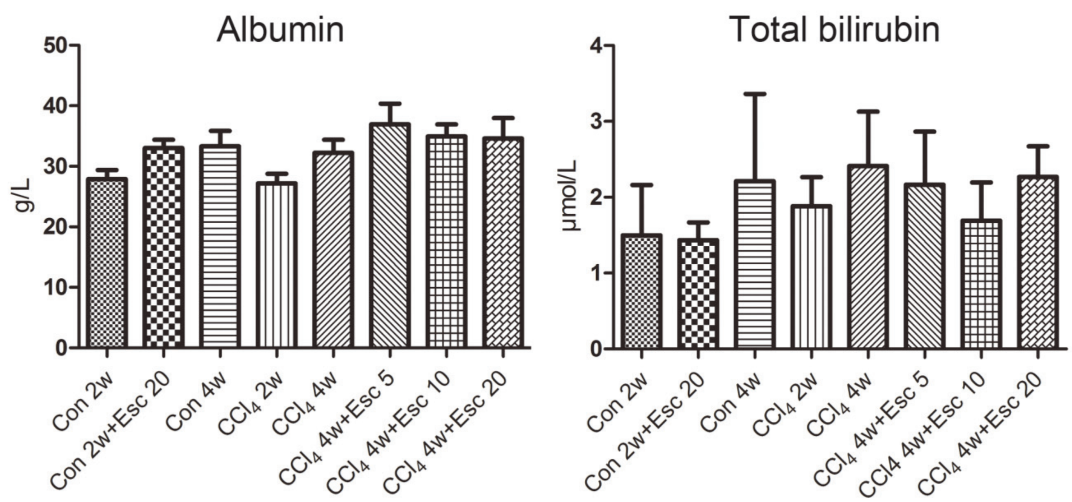
Supplementary Figure S1. Esculetin does not induce necrosis in PMF or HSC. Primary rat PMF (white bars) and HSC (black bars) were cultured for 3 days in the absence or presence of esculetin (see also Fig. 1) and the LDH activity present in the medium was determined.

4

Methods:

Cell necrosis measurement

The lactate dehydrogenase (LDH) release assay was used to determine necrotic cell death. Primary HSC and PMF were cultured for 3 days in the absence or presence of esculetin. Medium was harvested and LDH activity was determined by monitoring the oxidation of NADH to NAD parallel to the conversion of pyruvate to lactate [19]. The oxidation of NADH was measured at 340 nm for 30 min with 1 min interval at 37°C using a Bio-Tek EL808 Thermo microplate reader (Bio-Tek). The linear portion of the kinetic curve was used to calculate LDH activity when compared to a standard curve.



Supplementary Figure S2. CCl_4 and/or esculetin treatment do not change serum albumin and total bilirubin levels in mice. Mice were treated with CCl_4 with or without esculetin co-treatment as indicated (see also Fig. 2). Serum albumin and total bilirubin levels were determined at sacrifice. No significant differences were detected between treatment groups and control mice.

Methods:

Biochemical analysis

Serum albumin and total bilirubin were quantified by Bio-sinew kits (Chengdu, China) according to the suppliers' protocol using an automatic chemistry analyzer (Accute TBA-40FR, Toshiba Medical Systems Corporation, Japan).

Chapter 5

Short and early treatment with esculetin effectively ameliorates CCl_4-induced liver injury and fibrosis in mice

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Manuscript in preparation

Abstract

Background: Liver fibrosis develops as a result of chronic liver injury, as in viral hepatitis, autoimmune hepatitis, alcoholic and non-alcoholic steatohepatitis ((N)ASH). Fibrosis is a result of excessive extracellular matrix production, including collagens, by hepatic myofibroblasts. Fibrosis is largely reversible, but when liver injury persists may progress to cirrhosis and hepatocellular carcinoma. No drug-based therapy is available to treat liver fibrosis. Esculetin is present in many medicinal plants and harbors hepatoprotective properties. Here, we analyzed the optimal timing and delivery-route to ameliorate CCl_4-induced liver injury and fibrosis in mice.

Methods: Kunming mice were treated for 4 weeks with CCl_4 with 1-week esculetin co-treatment either in week 2, week 3 or week 4. Esculetin was administered by i.p. injection (10 mg/kg for 7 consecutive days) or by i.v. injection (6 mg/kg for 5 consecutive days). Animals were sacrificed 48 h after the last CCl_4 injection. Liver tissue was harvested for histological analysis, protein and RNA isolations, and serum samples were collected for biochemical parameters.

Results: CCl_4 strongly induced serum transaminases (AST and ALT) and hepatic Collagen 1a1 and a-SMA expression in mice. Esculetin most effectively suppressed CCl_4-induced AST and ALT when it was administered in the 2nd week of the 4-week CCl_4 treatment. Intravenous esculetin most effectively reduced *Collagen1a1* and *Acta2* (encoding a-SMA) expression; the earlier it was administered the stronger the suppression of fibrotic markers. Collagen deposition was reduced in mice treated with esculetin in the 2nd week of the 4-week CCl_4 liver toxicity model.

Conclusions: Esculetin provides long-lasting protection against CCl_4-induced liver injury and fibrosis in mice and this natural compound is therefore a relevant drug candidate for the prevention and treatment of chronic liver disease.

Introduction

Chronic liver diseases, such as viral hepatitis, autoimmune hepatitis and (non-)alcoholic liver disease ((N)ASH), invariably lead to liver fibrosis [1-3]. Liver fibrosis develops due to an uncontrolled wound healing response to chronic injury and is characterized by the deposition of excessive amounts of extracellular matrix proteins, particularly collagens and fibronectins, between hepatic cells. This impairs blood flow through the liver and disrupts the hepatic architecture resulting in reduced liver function and portal hypertension. Damage to the liver leads to the production of pro-inflammatory cytokines, chemokines, growth factors and reactive oxygen species. These conditions induce the transdifferentiation of quiescent hepatic stellate cells (HSC) into migratory and proliferative myofibroblasts that produce excessive amounts of extracellular matrix proteins and are characterized by high expression of α-smooth muscle actin (α SMA) [4, 5]. Both animal and human studies have shown that fibrosis is largely reversible and that, upon removal of the underlying cause, hepatic fibrosis resolves, reestablishing a near-normal liver architecture. However, upon continued liver injury, fibrosis may progress to cirrhosis, where the excessive ECM deposition leads to irreversible scarring of the liver that can cause liver failure and predisposes to liver cancer [6, 7]. Hepatocellular carcinoma is a leading cause of liver-related mortality in patients with cirrhosis [8-10]. At present, liver transplantation is the only available treatment for liver failure and primary liver cancer. Thus, there is a need for drug-based therapies that ameliorate and preferably reverse liver fibrosis. A critical parameter for the clinical success of effective anti-fibrotic drug is not only its potency to prevent, suppress and/or reverse liver fibrosis, but also that single doses (or short treatment periods) have long lasting effects.

Esculetin, or 6,7-dihydroxycoumarin, is present in many different plants with medicinal properties, including *Fraxinus rhynchophylla*[11], *Artemisia scoparia*, *Artemesia capillaris*, and in the leaves of *Citrus limonia* [12, 13]. Various therapeutic properties have been assigned to esculetin, including anti-antioxidant, anti-inflammatory and anti-tumor activities [14-16]. With respect to liver disease, esculetin has been shown to protect against acute [17, 18] and chronic [19] liver injury and it suppresses the development of liver cancer as well [20, 21]. Esculetin is a non-competitive inhibitor of lipoxygenases (5-lipoxygenase (5-LO) and 12/15-LO) and inhibition of 5-LO suppresses HSC proliferation and activation [22]. We have recently shown that esculetin strongly suppresses HSC proliferation and activation *in vitro* as well as CCl_4-induced liver fibrosis in C57BL/6 mice (Chapter 4). Esculetin was given daily intraperitoneally (i.p.) in the final 2 weeks of a 4-week CCl_4 treatment and completely blocked fibrosis progression as established after the first 2 weeks of CCl_4 treatment (Chapter 4). In this follow-up study, we aimed to determine whether 1) esculetin also ameliorates CCl_4-induced liver fibrosis in a different mouse strain 2) short term esculetin treatment reveals long-lasting anti-fibrotic effects and 3) intraperitoneal (i.p.) application of esculetin, as used in our preceding study, is superior to intravenous (i.v.) application of this compound.

In order to answer these questions, Kunming mice received 2 CCl_4 injections per week for 4 weeks to induce liver fibrosis. Esculetin was given i.p. or i.v. every day in week 2, or in week 3, or in week 4 of the CCl_4 treatment (Fig. 1). Mice were sacrificed 2 days after the final CCl_4 treatment at the start of week 5 and blood and liver tissue were analyzed for liver damage and fibrosis markers. Our data show that a 1-week esculetin treatment is most effective in preventing hepatocyte damage and liver fibrosis when given intravenously to mice early in a 4-week CCl_4 exposure protocol.

Material and methods

Animals, model of CCl_4-induced liver fibrosis and esculetin treatment regimes

Male Kunming mice (18~22g, age 6-8 weeks) were provided by the laboratory animal center of the Shantou University Medical College and hosted in a SPF animal facility under standard 12hr light/12hr dark cycle and fed standard rodent chow and water ad libitum. After 7 days adjustment, mice were randomly divided into eight treatment groups (See Fig. 1; one control group, seven CCl_4-treatment groups, six of which received 1-week esculetin treatment (i.p. or i.v.) in week 2, or week 3, or week 4. All groups contained 5 animals. CCl_4-treatment to induce liver fibrosis was performed as previously described [23] by twice-weekly injections of CCl_4 (i.p.; 0.4 μl/g BW) diluted 1:3 in olive oil CP (Aladdin Chemistry Co. Ltd, Shanghai, China) for 4 weeks (total 9 injections). Esculetin treatment was performed either in week 2, or week 3 or week 4, applied either by i.p. injection (10 mg/kg for 7 consecutive days) or i.v. injection (6 mg/kg for 5 consecutive days). Sham control mice only received olive oil. Mice were sacrificed 48 h after the last CCl_4 injection. Blood samples were collected at sacrifice for biochemical analyses. The left lateral liver lobe was prepared for histological analyses. Remaining liver tissue was snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein isolation.

Serum biomarkers

Alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and albumin (Alb) were measured using Flex reagent cartridge (Siemens Healthcare diagnostics Inc. Newark, USA) specific for Integrated Chemistry System (Dimension RxL Max, Siemens).

RNA Isolation and Q-PCR

Total RNA of liver tissue was isolated, purified and reverse transcribed following the protocol of the manufacturer (TaKaRa, Japan). Quantification of reverse-transcription polymerase chain was performed using SYBR green (TaKaRa) on the Eco Real-Time PCR System (Illumine USA), each sample in duplicate wells. Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (Ct value) were normalized to the endogenous control (*Gapdh*) and correlated inversely with initial mRNA levels. Relative quantification was performed using the ddCT method. All primers applied were synthesized by BGI (Shenzhen, China), as follows: *Acta2* forward primer: 5′-GGA GAA GCC CAG CCA GTC GC-3′; reverse primer: 5′-AGC CGG CCT TAC AGA GCC CA-3′. *Collagen 1a1* forward primer: ACT TCA GCT TCC TGC CTC AG; reverse primer: TGA CTC AGG CTC TTG AGG GT. *Alox12/15* forward primer: ATG GTG CTG AAG CGG TCT AC; reverse primer: ATC CGC TTC AAA CAG AGT GC. *Gapdh* forward primer: 5′-GCA CAG TCA AGG CCG AGA AT-3′; reverse primer: 5′-GCC TTC TCC ATG GTG GTG AA-3′.

Western blot analysis

Liver samples were homogenized in RIPA lysis buffer (P0013B; Beyotime). Equal amounts of proteins (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose filter membranes. Following blocking, membranes were probed with primary antibodies: mouse anti α-SMA monoclonal antibody 1:2,000 (Sigma), and mouse anti-GAPDH monoclonal antibody 1:3,000 (ZSGB-BIO, Beijing), followed by HRP-conjugated secondary mouse antibody 1:60,000 or secondary rabbit antibody 1:80,000 (ZSGB-BIO, Beijing) at room temperature for 1 h. Target proteins were visualized using the enhanced chemiluminescence SuperSignal West Dura detection system (Thermo Scientific, IL, USA). Signals were quantified by Quantity One (Bio-Rad).

Histology and immunohistochemical studies

Liver samples were fixed in 4% paraformaldehyde and embedded in paraffin. 4-μm paraffin sections were processed following routine methods. Masson trichrome and Sirius-red staining were performed according to manufacturer’s instructions (Beijing Leagene Biotechnology Co. Ltd.).

Statistical Analysis

All data are presented as mean ± standard deviation. Significance of differences between groups was tested by one-way ANOVA and t-test. Calculations were made using the software of GraphPad Prism 5. Results were considered statistically different when the p value <0.05.

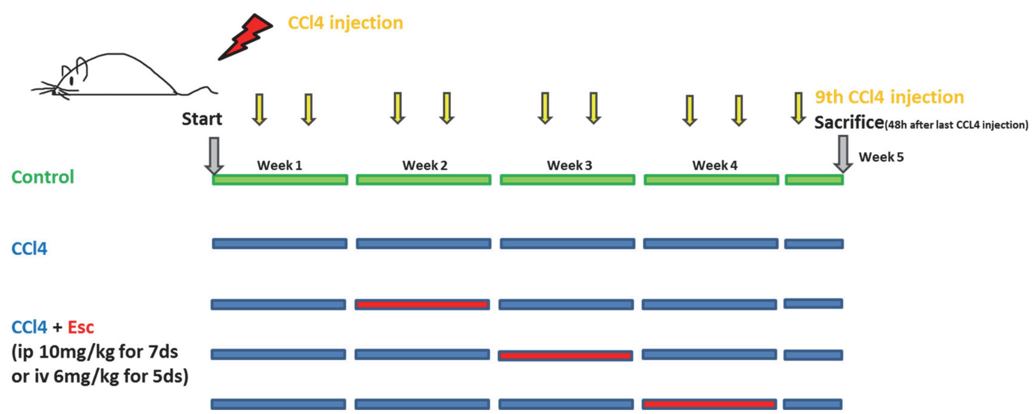

Figure 1. Overview of the experimental groups. Male Kunming mice were randomly divided into 8 groups (n=5). Control group (green bars) received olive oil as vehicle control. CCl_4-treated groups (blue bars) received CCl_4 (i.p. 0.4 ul/g b.w.) diluted 1:3 in olive oil and injected twice a week for 4 weeks (total 9 injections). Esculetin treatment groups (red bars) received esculetin administered i.p. (10 mg/kg for 7 consecutive days) or i.v. (6 mg/kg for 5 consecutive days) during the second, third and fourth week of CCl_4 challenge. Animals were sacrificed 48 h after the last CCl_4 injection.

Results

Early esculetin therapy effectively suppresses liver damage caused by continued CCl_4 treatment.

Chronic treatment with CCl_4 caused a strong increase in serum AST and ALT levels 48 h after the final (9th) CCl_4 injection in Kunming mice in comparison to sham-treated control mice (Fig. 2 A and B). Intraperitoneal (CCl_4+Esc p3) or intravenous (CCl_4+Esc v3) esculetin treatment in the 4th week did not significantly reduce the ALT and AST levels though trends towards a reduction in the transaminases were observed. In contrast, esculetin treatments, either i.p. or i.v., in the 2nd or 3rd week of the CCl_4 protocol significantly and strongly reduced ALT and AST levels in the serum of these mice. ALT levels were reduced by 61% (i.p) and 72% (i.v.) when mice were treated with esculetin in the 3rd week, while the reduction of these liver damage markers was even more pronounced when the CCl_4-treated mice were co-treated with esculetin in the 2nd week (-73% for i.p. and -81% for i.v.; Fig. 2A) Similar data were obtained for AST (Fig. 2B), where both treatment regimens (i.p. and i.v.) given in the 2nd week completely prevented the CCl_4-induced increase at the end of the 4-week protocol. A 4-week CCl_4 treatment did not affect the serum albumin levels, which was also not further changed by the various esculetin treatments (Fig. 2C). Similarly, also serum LDH levels were hardly changed by CCl_4 and the esculetin co-treatments (Fig. 2D).

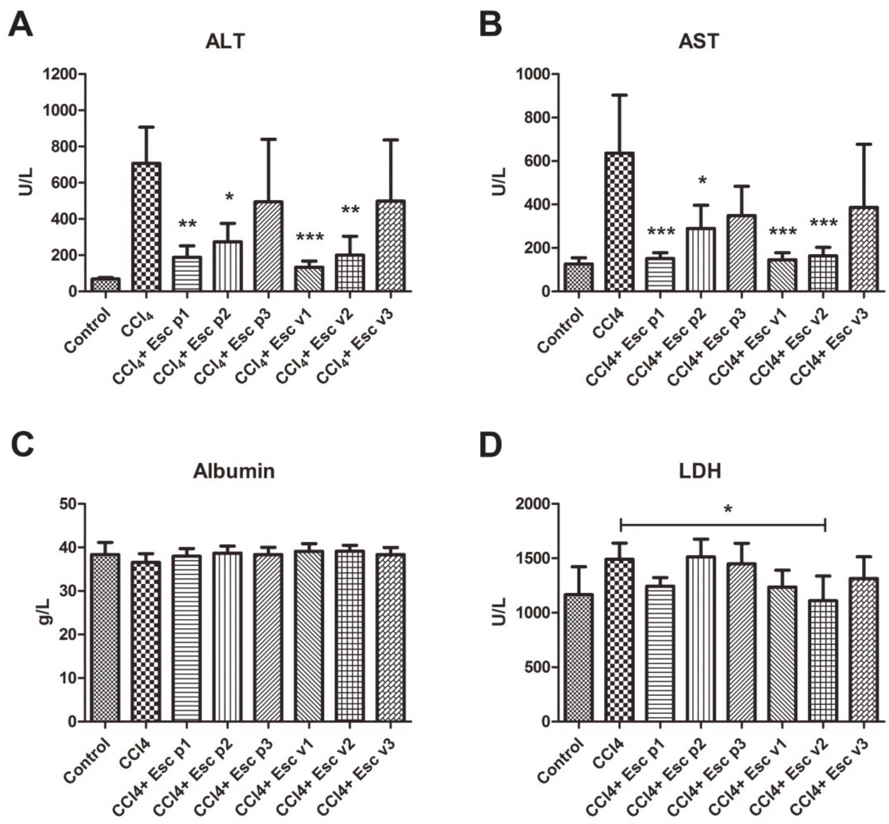

Figure 2. Early esculetin treatment effectively suppresses serum liver injury markers in CCl_4-treated Kunming mice. Serum liver damage and function markers at sacrifice: A) alanine transaminase (ALT), B) aspartate transaminase (AST), C) albumin (Alb) and D) lactate dehydrogenase (LDH). **P* ≤ 0.05, ** *P* ≤ 0.01, *** *P* ≤ 0.001.

Early intravenous esculetin therapy effectively suppresses fibrotic marker expression in CCl_4 treated mice.

Acta2 (encoding α-SMA) and *Collagen 1a1* expression was strongly (2.2-fold and 14-fold, respectively) induced in 4-week CCl_4-treated Kunming mice compared to sham-treated animals (Fig. 3A, B). Intravenous therapy with esculetin in week 2 significantly reduced expression of these markers of fibrosis, and *Acta2* expression was even back to control levels. Intravenous esculetin treatment in week 3 or in week 4 did not significantly reduce *Acta2* and *Coll1a1* expression although a trend towards reduced expression was observed. Intraperitoneal esculetin therapy suppressed *Coll1a1* expression most effectively when given in week 3 (-55%). A trend to lower *Acta2* expression was also observed in this therapy group, although not reaching significance. Intraperitoneal esculetin treatments in week 2 or week 4 did no lower *Acta2* and *Coll1a1* expression level. Hepatic α-SMA protein levels were strongly increased (4.5-fold) upon 4-week CCl_4-treatment and early treatment with esculetin in week 2, either i.p. or i.v., significantly reduced the level of this maker of fibrosis (-44% and -48%, respectively; Fig. 3C). Esculetin is a non-competitive inhibitor of lipoxygenases and previously we found that esculetin therapy in the final 2 weeks of a 4-week CCl_4 treatment period fully suppressed the CCl_4-induced *Alox12/15* mRNA expression in C57BL/6 mice (Chapter 4). CCl_4 also strongly induced *Alox12/15* expression in Kunming mice (27-fold), which was partly suppressed to a similar extent in all esculetin treatment groups by approximately 60% (Fig. 3D).

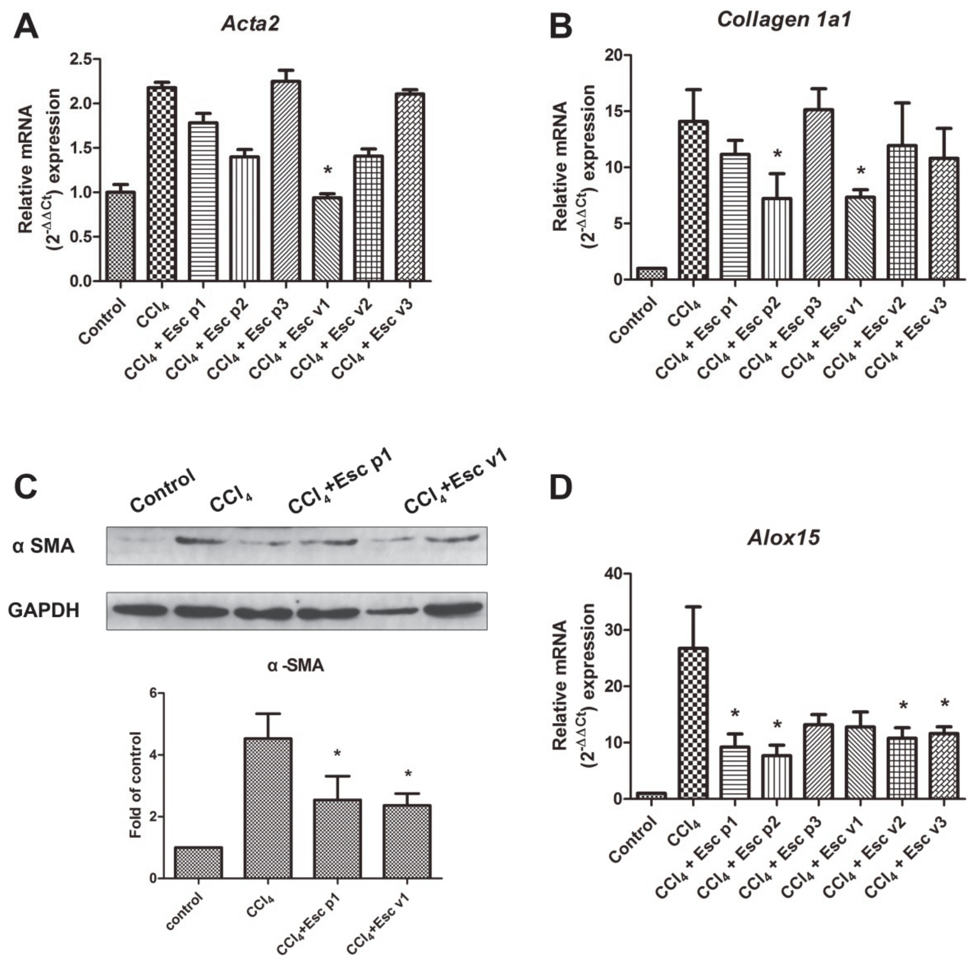

Figure 3. Early intravenous esculetin treatment most effectively suppresses CCl4-induced expression of markers of fibrosis. Liver homogenates were analyzed for mRNA levels of A) Acta2, B) Collagen 1a1 and D) Alox12/15. C) α-SMA protein expression was determined by Western blotting, using GAPDH as loading control. Protein bands were quantified by densitometry and corrected for GAPDH.* P ≤0.05.

Early esculetin treatment suppresses collagen deposition in CCl_4 treated mice.

Based on the results described above, we selected the groups that were treated with esculetin in week 2 for further histological analysis. Masson's trichrome staining for collagen showed that the 4-week CCl_4 treatment induced significant liver fibrosis in

Kunming mice, as compared to mice treated with the vehicle alone (Fig. 4A and B). Collagen deposition was particularly detected in the pericentral area and periportal regions, resulting in bridging fibrosis. Some pericellular fibrosis was also observed. Esculetin treatment in week 2, either i.p. or i.v., significantly reduced collagen deposition in the 4-week CCl_4 treated mice (Fig. 4C and D). Some diffuse pericentral and periportal collagen was still observed, but bridging fibrosis was absent.

Collectively, these data show that early, transient esculetin therapy significantly reduces liver injury and liver fibrosis development in Kunming mice treated for 4 weeks with CCl_4.

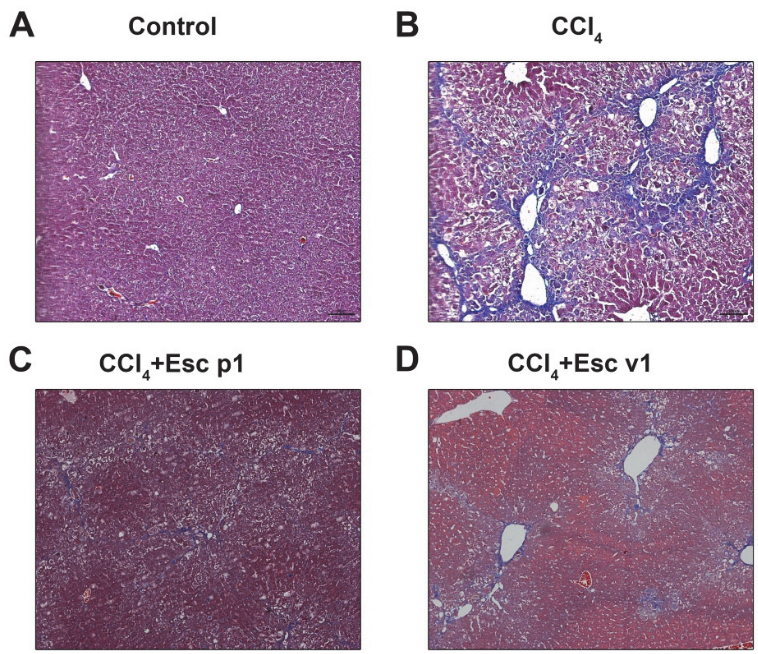

Figure 4. Early esculetin treatment decreases hepatic collagen deposition. Collagen deposition was determined by Masson trichrome staining (original magnification 200×) in control mice, 4-week CCl_4 treated mice; and mice treated in de 2nd week with esculetin either i.p. (CCl_4+Esc p1) or i.v. (CCl_4+Esc v1).

Discussion

In this study, we compared the hepatoprotective effect of a short esculetin treatment period at different time points during CCl_4 treatment. We show that a short esculetin intravenous treatment period for 5 consecutive days (i.v.; 6 mg/kg each) in week 2 was most effective in suppressing serum liver damage markers, as well as liver fibrosis markers at the end of the CCl_4 treatment period of 4 weeks, when compared to esculetin treatment in week 3 or 4. Thus, early treatment with the natural compound esculetin has long-lasting therapeutic effects on the development of CCl_4-induced liver fibrosis in mice.

Esculetin is a natural compound found in many medicinal plants [11, 24, 25]. One of its molecular actions is the inhibition of lipoxygenases, enzymes involved in the inflammatory and fibrotic cascade [26-30]. We have previously shown that esculetin directly inhibits hepatic stellate cell proliferation and activation and arrests,-and in part reverses- the development of liver fibrosis when given in the final 2 weeks of a 4-week CCl_4 treatment of C57BL/6 mice (Chapter 4). A concentration-dependent anti-fibrotic effect was observed for esculetin when given in daily i.p. doses of 5, 10 and 20 mg/kg. In this follow-up study, a 4 week CCl_4 treatment was applied to Kunming mice and esculetin was given i.p. for 7 consecutive days at 10 mg/kg or i.v. for 5 consecutive days at 6 mg/kg. The 4-week CCl_4 treatment of Kunming mice resulted in highly comparable elevation of liver damage markers AST and ALT as well as liver fibrosis markers as compared to C57BL/6 mice, confirming that this outbred mouse strain that is derived from Swiss albino mice and is commonly used in China as a laboratory animal is a good model to study CCl_4-induced liver toxicity. Indeed, similar observations have recently been reported by others, both using acute [31-34] and chronic CCl_4 models [35, 36].

The 2 week esculetin treatment in the 2nd part of the 4 week CCl_4 protocol in C57BL/6 mice did not affect *Alox5* expression, while the CCl_4-induced *Alox12/15* mRNA levels (+23-fold) were fully suppressed to control levels in to C57BL/6 mice (Chapter 4). *Alox 12/15* mRNA levels were similarly enhanced in 4-week CCl_4-treated Kunming mice (+27-fold), but only partly reduced (~50-60%) by a short esculetin treatment in this study. Remarkably though, very similar reductions in *Alox 12/15* mRNA levels were observed in all treatment groups, independent of application method (i.p. or i.v.) and moment of treatment (in 2nd, 3rd or 4th week of CCl_4 exposure). Although the *Alox 12/15* mRNA level is merely a marker for the inflammatory/fibrotic signaling cascade and not for the level of activity as result of the esculetin-mediated inhibition, it does indicate that the length of the esculetin treatment determines the level of suppression of *Alox12/15* expression and is independent of the timing within the 4-week CCl_4 protocol. In contrast, early esculetin treatment, either i.p. or i.v., had the most pronounced effect on hepatocyte injury markers (AST and ALT) and appeared less effective when given at later moments in the 4-week CCl_4 protocol. This suggests that the hepatoprotective effect of esculetin requires a relatively long period to reach its full potential. A direct hepatoprotective effect of esculetin has been reported in acute models of liver injury, including models of a single dose of CCl_4 or paracetamol in rats [18, 37-39], which is particularly relevant for therapeutic application in acute liver disease. The long-lasting hepatoprotective effect of esculetin observed in this study may be even more relevant as it may protect against acute challenges to the liver at later time points. The pharmacokinetics of esculetin have not been analyzed in this study, but a recent study found that it quickly distributes to the liver and kidneys of rats after oral intake [40]. Plasma levels peaked already after 5 minutes upon oral administration and dropped to undetectable levels 3 h post-administration, at which time point significant amounts remained detectable only in liver and renal tissue. In light of these results, it is now highly relevant to determine whether esculetin remains present in the liver and if so in which liver cells, in the period after the last dose.

Although apparently equally protective against hepatocyte injury as i.p. administration, early i.v. administration of esculetin appeared to be more potent in suppressing markers of liver fibrosis (*Acta2* and *Coll1a1* expression). This was not related to the total esculetin exposure, since the total exposure was actually more than 2-fold lower in the i.v. therapy group (5 days x 6 mg/kg=30 mg esculetin /kg bw in the i.v. group vs. 7 days x 10 mg/kg = 70 mg esculetin/kg bw in the i.p. group). Obviously, these differences warrant further investigation, but it suggests that the administration route of esculetin affects its therapeutic potential.

In conclusion, we show that esculetin, a natural product of medicinal plants, has potent and long-lasting therapeutic effects on CCl_4-induced liver injury and fibrosis in mice, which warrants future studies on establishing the molecular mechanisms involved and translating this knowledge to clinical studies in humans.

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Chapter 6

General discussion and future perspectives

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Over the past decades, numerous studies have addressed the treatment of chronic liver diseases, identifying a growing number of promising molecules and therapeutic interventions in experimental models, although the step towards systematic clinical studies has yet to be made. In the final chapter of this thesis, we will discuss the results of our studies relative to the current state of knowledge on liver inflammation and hepatic fibrosis.

Liver inflammation

Programed cell death is an essential process in normal physiological processes and tissue homeostasis. However, dysregulation of cell death, in particular the apoptotic program is a sign of stress, injury or infection and is linked to tissue damage and disease pathogenesis. In liver diseases, different forms of cell death occur, depending on the liver disease (as discussed in **Chapter 1**). In addition to hepatocytes, cell death also affects non-hepatocytic cell types, like cholangiocytes [1, 2], hepatic stellate cells [3] and sinusoidal endothelial cells [4, 5]. However, hepatocyte death is a key event in the progression of liver diseases. Although hepatocyte cell death can be considered as a normal and beneficial event to remove damaged or malignant cells, excessive and prolonged hepatocyte cell death leads to loss of liver function, chronic inflammation, fibrosis and, because of the continuous regeneration, hepatocellular carcinoma Therefore, the contribution of cell death to the progression of liver diseases is cell-, stage- and context-specific [6]. From animal studies it has been concluded that acute liver failure is characterized by both severe apoptosis and necrosis; cholestatic liver diseases and some drug-induced liver failure, like acetaminophen (APAP, paracetamol) intoxication are characterized mainly by necrotic cell death [6-8] and cell death in NASH is characterized by moderate apoptosis, although human studies also demonstrated necroptosis [9]. In many liver diseases both necrosis and apoptosis occur simultaneously, e.g. in ALD and NAFLD [10-14].

Massive cell death is a hallmark of acute liver diseases and leads to a dramatic loss of liver function. Therefore, specific interventions targeted to prevent or attenuate this massive cell death may be very effective in these acute conditions. Caspase inhibitors like IND-6556 and GS-9450 have been investigated and effectively block the apoptotic cascade and reduce liver injury in experimental models [15-18]. Unfortunately, inhibition of caspase-dependent apoptosis may lead to caspase-independent apoptosis, necroptosis or necrosis and therefore caspase inhibitors have not achieved widespread application in clinical hepatology [6]. Initially, cell death was considered to be the result of inflammation, e.g. as a result of the generation of reactive oxygen species, however, cell death can also precede, trigger or amplify the inflammatory response directly by disrupting epithelial barriers and triggering immune responses [19]. Hence, inhibition of inflammation is considered an effective strategy in reducing liver injury. Inflammation is a complex process in which numerous cell types, organelles and signaling factors are involved, e.g. Fas/FasL, TNF/TNF-receptor, Damage-Associated Molecular Patterns (DAMPs), chemokines, MAP-kinases, transcription factors like Nuclear Factor-κB (NF-κB), mitochondrial pathways etc. NF-kB has emerged as a central regulator of inflammatory activity. Inhibition of NF-κB or NF-κB target genes is considered an attractive strategy for anti-inflammatory therapy and caffeic acid, curcumin, resveratrol and silymarin have been used as NF-κB inhibiting interventions [20]. Alternatively, since TNF is both a NF-κB-dependent gene in inflammatory cells as well as an inducer of NF-κB in target cells, anti-TNF therapy is also considered as an anti-inflammatory intervention. However, all TNF-antagonists currently used, e.g. Infliximab, have also been associated with drug-induced liver injury [21, 22]. This is probably due to the lack of target-cell specificity of TNF-antagonists: TNF antagonists will also prevent NF-κB activation in epithelial cells like hepatocytes and in these cell types, NF-κB acts as a survival factor [23]. Interestingly, TNF-R knockout mice were protected from hepatic steatosis and fibrosis [24]. In addition to TNFα/NF-κB antagonists, other compounds have been evaluated as anti-inflammatory agents in liver inflammation. E.g. glycyrrhizin, used as inhibitor of both DAMP and HMGB-1 showed a beneficial effect in HCV and HCC [25-27]. Depletion of Kupffer cells is also effective in the prevention of inflammation and subsequent liver injury [28-30]. To summarize, therapy may be aimed to directly inhibit cell death of hepatocytes and/or indirectly via inhibition of inflammatory pathways. Furthermore, anti-inflammatory interventions may have opposite effects in different cell types, therefore, cell-specific targeting may be necessary to achieve optimal results. In view of these considerations, the raw extract of *Ipomoea stolonifera* may be a promising candidate as discussed below.

In **Chapter 2,** we demonstrate that the raw extract of *Ipomoea stolonifera* and its purified components profoundly reduce caspase-3 activation in hepatocytes exposed to the hydrophobic bile acid glycochenodeoxycholic acid (GCDCA). Since activation of caspase-3 is a late event in the apoptotic pathway and invariably leads to cell death, the prevention of caspase-3 activation demonstrates a truly protective effect of BE-IS, the raw extract of *Ipomoea stolonifera* and its components. In addition to the cytoprotective effect, the anti-inflammatory properties of BE-IS and its components were also assessed. Hepatocyte inflammation was induced by a combination of three inflammatory cytokines (TNFα, IL-1β and IFN-γ). Our results provide evidence for the anti-inflammatory action of BE-IS and some of its components. Of note, the anti-inflammatory effect is independent of the cytoprotective effect, since the GCDCA-induced model of hepatocyte death is not related to inflammation [31]. BE-IS contains multiple bioactive components and has been demonstrated to have therapeutic effects in the management of various liver diseases [32]. Very recently, we further investigated the synergistic effect and dose optimization of the five main components of BE-IS used in **Chapter 2,** *in vivo* and *in vitro* [33]. It was demonstrated that esculetin, curcumin and hesperetin are the principal constituents having synergistic effects. Combined, our findings demonstrate that BE-IS (or a selected combination of its main components) has a superior effectiveness over single-agent therapy with respect to cytoprotection and attenuation of inflammation. On the other hand, the single components should also be evaluated in more detail. E.g. curcumin has been demonstrated to be effective in attenuating inflammation via inhibition of TNFα, NF-κB and COX-2 [34-36]. Additional studies in relevant *in vivo* models will be needed to assess the potency of these natural compounds against liver inflammation.

Liver macrophages (resident macrophages or Kupffer cells) and hepatic leukocytes (NK cells and NKT cells) play a crucial role in the first line of defense against external and internal pathogens and irritants. These cells can rapidly produce copious amounts of cytokines after activation, which enables them to respond quickly and subsequently help to remove invading pathogens, toxins and food antigens. However, if this defense system is inadequately activated, severe inflammation, ultimately resulting in septic shock and multiple organ failure may occur, with cells of the innate immune system contributing to and amplifying the liver injury [37-39]. Hence, it is hypothesized that drugs that affect immune function may be beneficial in the treatment of liver diseases. In the liver, various bacterial stimuli can activate Kupffer cells, e.g. lipopolysaccharide (LPS), the cell wall component of gram negative bacteria. LPS directly activates Kupffer cells and leads to the production of large amounts of cytokines, such as interleukins, chemokines etc. Of these cytokines, TNFα plays a crucial role, since it plays a key role in liver inflammation and, under specific conditions, is also a potent inducer of apoptosis [40].

In **Chapter 3**, we used two *in vivo* models, e.g. lipopolysaccharide (LPS)-induced and Concanavalin A (Con A)-induced fulminant hepatitis, to analyze the anti-inflammatory properties of one of the purified components from *Ipomoea stolonifera* raw extracts, namely hesperetin. These two models differ with respect to the initiating mechanism and the main cell types and cytokines involved in the onset and perpetuation of fulminant hepatitis. E.g., unlike LPS-induced hepatitis, Con A-induced liver injury is dependent on T-cell mediated phenomena [41] and cannot be induced in athymic nude mice. Con A is therefore considered to be a model for human autoimmune hepatitis [42]. Con A is an activator of NKT cells. After Con A injection, NKT cells produce large amounts of cytokines such as IFNγ, IL-4, IL-13, TNFα and GM-CSF [43]. Con A-induced cytokines, such as TNFα and IFNγ, have a direct role in the induction of widespread hepatocyte apoptosis [18]. IFNγ, a signature cytokine of NK/NKT cells inhibits hepatocyte proliferation and regeneration in liver injury [44]. Moreover, NKT cells cause hepatocyte cell death by releasing pro-apoptotic FasL [45]. LPS or endotoxin is a cell wall product of Gram-negative bacteria and a very potent activator of macrophages and inflammation. Exposure of macrophages, including Kupffer cells, to LPS leads to increased generation of inflammatory cytokines like TNFα, IL-1 and Il-6, as well as chemokines and reactive oxygen species. Uncontrolled exposure to LPS/endotoxin, as happens in sepsis, can lead to septic shock and death. Since LPS is also a potent inducer of IFNγ, Kupffer cells are also able to activate liver NK and NKT cells [46].

Our data provide compelling evidence for the hepatoprotective effects of hesperetin in LPS-induced and Con A-induced fulminant hepatitis, which is underscored by its ability to reduce TNFα-depended apoptosis via the JNK pathway and dramatically repress IFNγ expression. The clinical efficacy of anti-TNF therapy has been established in chronic inflammatory diseases like inflammatory bowel disease (IBD), and rheumatoid arthritis [47]. Some studies have suggested that Con A-induced hepatitis can be inhibited by treatment with antibodies against IFNγ or IL-4 [48, 49]. In another study, LPS-induced hepatic injury in *Propionibacterium acnes*-primed mice was completely blocked by the combination of anti-IFNγ and anti-TNFα therapy and only partially blocked by inhibition of only one pathway [42]. Therefore, hesperetin, which is able to inhibit both IFNγ and TNFα holds great promise for the treatment of inflammatory diseases, including liver failure. Hesperetin is abundant in citrus fruits and its daily consumption can be high [50, 51]. In chapter 3, we demonstrated the remarkably protective effect of orally ingested hesperetin in fulminant hepatitis. Given the bioavailability of hesperetin when taken orally and the proven safety and tolerability, hesperetin is a promising candidate for the treatment of both acute and chronic liver diseases.

Liver fibrosis

Liver fibrosis is a dynamic and, at least to a certain extent, reversible process which involves many liver cell types and numerous mediators (described in detail in **Chapter 1**). Treatment of chronic liver diseases, including liver fibrosis has been hampered by the lack of effective therapeutic interventions. Early liver fibrosis, with absence of extensive ECM crosslinking and marked angiogenesis can still reverse to near-normal architecture when the underlying cause is successfully treated or removed (e.g. viral eradication or cessation of alcohol abuse). Chronic inflammation inevitably leads to liver fibrosis and is characterized by injury and damage of hepatocytes, persistent activation of inflammatory cells, including Kupffer cells and increased production of ECM by activated hepatic stellate cells, regardless of the etiology. Many of these effects are caused by inflammatory cytokines, chemokines and oxidative stress, resulting in death (apoptosis, necrosis) of the functional hepatocytes. Therefore, an effective treatment for liver fibrosis should block the action of these noxious factors and/or cell death of hepatocytes [52, 53], and should reduce the inflammatory state of macrophages, enhance the anti-oxidant status of hepatocytes and reduce steatosis through lipogenic pathways [54-59]. Importantly, therefore, drugs that address more than one single pathogenic pathway will be more efficient than drugs that target only a single pathway [60].

To determine the efficacy of esculetin as an antifibrotic drug, in **Chapter 4**, we studied the direct effect of esculetin on cultured rat HSC, and subjected mice to the established chronic model of CCl_4-induced fibrosis, where mice were given esculetin treatment during the final 2 weeks of a 4 week CCl_4 treatment. This study revealed multiple antifibrotic properties of esculetin, including suppression of hepatic stellate cell activation, profibrotic TGF-β/Smad signaling and induction of fibrogenolysis by enhancing the MMP/TIMP-1 balance. Additionally, the anti-oxidant capacity of the liver is improved by increasing the hepatic GSH/GSSG ratio.

Indeed, many different aspects are involved in the process of fibrogenesis, e.g. organ inflammation, myofibroblast activation, scar tissue formation and fibrosis resolution. Therefore, in theory, molecular targets on any of this aspect will affect fibrosis progression. Ideally, the more targets, the better. In our study, esculetin seems to be such a promising anti-fibrotic agent that is multifaceted in various ways. HSC play a crucial role in hepatic fibrogenesis. HSC transdifferentiate into hepatic myofibroblasts and the excretion of excessive amount of ECM is the key event in fibrogenesis. Thus, suppression of proliferation and activation of hepatic myofibroblasts is a major focus of anti-fibrotic therapy. Esculetin strongly suppresses HSC proliferation and the expression of markers of fibrosis (*collagen 1a1* and α-Sma). Among different signaling events, the TGF-β/Smad pathway is a key profibrotic pathway associated in liver fibrosis. In our study, esculetin significantly inhibits *Tgf-β* expression and downstream Smad2/3 phosphorylation, while *Pparγ* and *Pdgfr-β* expression were not changed.

An interesting option to accelerate the resolution of fibrosis is to increase matrix degradation, a process that is controlled by balancing the level of matrix metalloproteinases (MMPs) and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs), in particular myofibroblast-derived TIMP-1 [61]. Increasing the activity of MMPs and/or antagonizing TIMPs will benefit matrix degradation as demonstrated by Iredale *et al*. and Issa *et al*. [3, 62] in seminal studies on the resolution of liver fibrosis in rats by decreasing the production of TIMP-1. In addition, it has been postulated that reducing TIMP activity in the liver may actually have the dual advantage of promoting matrix degradation and inducing apoptosis of highly fibrogenic HSCs [63]. In our study, CCl_4-induced expression of Mmp2 and Mmp13 were not changed by esculetin, while Mmp9 was only moderately reduced. Most pronounced, however, was the strong suppression of CCl_4-induced TIMP-1 upon esculetin treatment. Thus, the esculetin-increased MMP/TIMP-1 balance favors fibrogenolysis.

Our results suggest that the anti-fibrotic action of esculetin acts, at least in part, through inhibition of lipoxygenases, e.g. 5-LO and/or 12/15-LO. This is in line with our earlier observations that activation of RORα by melatonin or a synthetic agonist (SR1078) suppresses 5-LO expression in HSC and thereby suppresses cell proliferation and expression of *Collagen1a1* and *Acta2* (encoding αSma) [64]. In a similar way, esculetin suppressed HSC proliferation and expression of those markers of fibrosis. Lipoxygenases (LOXs) are dioxygenases that catalyze the formation of corresponding hydroperoxides from polyunsaturated fatty acids, such as linoleic acid and arachidonic acid, to produce inflammatory lipid intermediates that directly or indirectly affect cellular function and survival [65]. Human and mouse express six LOX isoforms, which are defined by the site of oxygenation, either at carbon position 5, 12, or 15 [66]. Kupffer cells, the resident macrophages of liver, express *Alox5* and *Alox12/15*, while hepatocytes appear to be devoid of these enzymes [67-69]. Kupffer cells participate in the initiation of the inflammatory cascade leading to liver fibrosis by virtue of various proinflammatory factors, such as cytokines and leukotrienes, the latter being produced via the LOX pathway [70-72]. Thus, this pathway plays a direct role in HSC activation and possibly also modulating the extracellular matrix (ECM) in the liver [71]. Although the exact role of LOXs in liver fibrosis need to be established, inhibitors of 5-LO and/or 12-15-LO have been demonstrated to prevent hepatocellular injury during inflammation and liver fibrosis [67, 73, 74].

As we found that esculetin effectively blocks the progression of liver fibrosis even under continued liver toxicity conditions induced by CCl_4, we next aimed to analyze its therapeutic potential in short treatment periods through 2 different administration routes, namely via intraperitoneal or intravenous injections. A key consideration in the design of our study in **Chapter 5** was that esculetin treatment was started while liver injury and the development of fibrosis were already ongoing, most closely paralleling the situation in patients with (chronic) liver disease. Esculetin treatment was given only for 1 week, either in the 2nd, 3rd or 4th week of a 4-week CCl_4 protocol.

Remarkably, short early treatment of esculetin effectively suppressed liver injury (serum AST/ALT levels) and the progression of liver fibrosis. Intravenously administered esculetin was most effective in reducing serum transaminase (ALT/AST), HSC activation markers (Collagen 1a1 and α-SMA expression), as well as collagen deposition. So apparently, esculetin provides a potent and long-lasting therapeutic effect against CCl_4-induced liver injury and fibrosis in mice.

In the studies described in **Chapter 4 and 5**, esculetin was administered either i.p. or i.v. is this is expected to exert the strongest therapeutic effect. Since esculetin is present in many medicinal plants, it also relevant now to determine whether it has similar therapeutic properties as a nutritional supplement. Several studies have analyzed the safety and therapeutic properties of oral intake of esculetin. Long term (over months) dietary intake of esculetin is well-tolerated by mice and rats, with minimal side effects [75-77]. Moreover, Qin pi, the dried branch or stem bark of *Fraxinus rhynchophylla* that is rich in esculetin, is used in traditional medicine to treat patients with chronic bronchitis and bacillary dysentery in children [75]. In rodents, ingested esculetin is rapidly absorbed with peak plasma levels appearing within minutes after intake. Subsequently, esculetin rapidly disappears form the blood and accumulates in the liver and kidneys [78]. Thus, esculetin may hold promise in preventing livery injury and/or liver fibrosis as a nutritional supplement.

Future perspectives

Alternative and/or complementary therapy for the treatment of acute and chronic diseases has received increased attention and recognition over the past few decades. Various herbal extracts, as well as purified compounds from those extracts, show medical benefits in liver diseases and include phenolic compounds, terpenoids, alkaloids and flavonoids [79-81]. Thus, natural products that are contained in millions of botanicals and herbs are a rich source for drug development. Approximately 20% to 30% of patients with chronic liver diseases use herbal products [82, 83] and sales of e.g. silymarin reaches $180 million in Germany alone [84]. This thesis aimed to identify the therapeutic compounds from one such medicinal herb, *Ipomoea stolonifera,* and provide insight in the mechanisms by which it suppresses fulminant liver inflammation and liver fibrosis.

First, a natural product needs to demonstrate therapeutic potential in a particular disease, but also safety and effectiveness of traditional medicine products needs to be established, e.g. for hesperetin, esculetin and the raw extract of BE-IS in our study. Such compounds may be used therapeutically when the disease is already established, but perhaps even more interesting is their potential to prevent disease development altogether, as our studies on esculetin demonstrate. Ideally, such compounds should be included in the diet as a supplement to prevent liver injury and fibrosis. Future experiments should reveal such hepatoprotective action of esculetin when given as a dietary supplement before liver injury is induced.

Although clear therapeutic actions have been assigned to several herbal extracts, their composition is highly dependent on environmental conditions, such as climate, season and the location where the herb grows. Therefore, the same "product" can vary significantly from batch to batch [85, 86]. Consequently, the taxonomic identity of the source and the chemical identity, purity and stability of the constituents may vary. This is clearly unacceptable when used as a therapeutic and even as a dietary supplement. Standardization of the extracts and quality and safety control are therefore urgently needed, but not in place yet. Approval of each new submission must therefore be based on a thorough evaluation of its composition, its safety and toxicological profile, pharmacokinetics and metabolism and identification of its active ingredients [87, 88]. Ultimately, the challenge will be in the identification of the active constituents of the extracts and the controlled reconstitution of the most effective mixture of these purified components.

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Appendix

Nederlandse samenvatting

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Binnen de groep leverziekten wordt er onderscheid gemaakt tussen acuut leverfalen en chronisch leverfalen. Acuut leverfalen wordt gekenmerkt door massale celdood en gaat gepaard met functieverlies. Sterfte van hepatocyten, zowel via apoptose als necrose, is een belangrijk aspect van acuut leverfalen. Daarom zijn in deze groep interventies die de massale celdood kunnen voorkomen of verminderen waarschijnlijk effectief voor het behoud van leverfunctie. Chronisch leverfalen daarentegen leidt tot metabole verstoringen, o.a. van toxines en geeft irreversibele chronische leverschade. Dit gaat vaak gepaard met chronische ontsteking. Het gevolg en eindstadium van chronisch leverfalen is lever fibrosis en cirrose. Fibrogenese is in eerste instantie een beschermingsmechanisme van de lever als reactie op schadelijke stimuli en ter voorkoming van verdere beschadiging. Echter, opstapeling van extracellulaire matrix (type I en IV fibrillair collageen), ernstige leverschade en ongecontrolleerde ontsteking, zullen uiteindelijk leiden tot irreversibele lever fibrose en zelfs lever kanker. Het doel van dit proefschrift is om het effect van butanol-extract van de traditionele medicinale plant *Ipomoea stolonifera* (BE-IS) en vijf gezuiverde componenten van dit extract te onderzoeken in acute en chronische leverziekten, met name in inflammatoire leverziekten en lever fibrose *in vivo* and *in vitro*.

In **hoofdstuk 1** geven we een overzicht van de meest belangrijke celtypen van de lever en signaalcascades die van belang zijn in inflammatoire leverziekten. Tevens wordt een overzicht gegeven van traditionele geneeswijzen.

In **hoofdstuk 2** is het lever-beschermende mechanisme van BE-IS en haar vijf gezuiverde componenten onderzocht op de inflammatoire respons en galzout-geïnduceerde celdood in primaire hepatocyten *in vitro*. We tonen aan dat het ruwe extract van *Ipomoea stolonifera* en haar vijf gezuiverde componenten caspase-3 activiteit sterk verminderen in hepatocyten die zijn blootgesteld aan het hydrofobe galzout glycochenodeoxycholic acid (GCDCA). Aangezien activatie van caspase-3 een laat effect is van de apoptose-cascade en vrijwel altijd leidt tot celdood, is het voorkomen van caspase-3 activatie een beschermend effect van BE-IS en haar componenten. Naast het celbeschermende effect zijn ook de anti-inflammatoire eigenschappen van BE-IS en haar componenten onderzocht. Ontsteking in hepatocyten werd geïnduceerd door drie inflammatoire cytokines (TNFα, IL-1β en IFN-γ). Onze resultaten geven weer dat BE-IS en sommige van haar componenten, een anti-inflammatoire werking hebben.

In **hoofdstuk 3** hebben we twee muismodellen gebruikt voor fulminante hepatitis: het concanavalin A (Con A) model en het D-galactosamine/lipopolysaccharide (D-GalN/LPS) model, om de therapeutische eigenschappen van hesperetin, één van de gezuiverde componenten van *Ipomoea stolonifera*, te onderzoeken. Deze twee modellen verschillen van elkaar in pathogenese, maar ook wat betreft celtypen en cytokines die van belang zijn in de initiatie en onderhoudende fase van de fulminante hepatitis. Onze resultaten wijzen duidelijk in het richting van een lever-beschermend effect van hesperetin, zowel in LPS-geïnduceerde als in Con

A-geïnduceerde fulminante hepatitis. Hesperetin is namelijk in staat om TNFα-afhankelijke apoptose via de JNK signaaltransductie cascade te remmen, alsmede IFNγ expressie sterk te verminderen. Hesperetin lijkt dus een goede kandidaat voor de behandeling van zowel acute als chronische leverziekten, ook gezien de biologische beschikbaarheid van hesperetin bij orale inname en de bewezen veiligheid en tolerantie.

Om het effect van esculetin als anti-fibrose medicament te onderzoeken, hebben we in **hoofdstuk 4** ten eerste het directe effect van esculetin op stellaatcellen *in vitro* onderzocht, en ten tweede muizen blootgesteld aan het chronisch model van CCl_4-geïnduceerde leverfibrose, waarin muizen de laatste twee weken van de behandeling met CCl_4 ook met esculetin behandeld werden. We tonen aan dat esculetin meerdere anti-fibrotische eigenschappen heeft; onderdrukking van de activatie van stellaatcellen, vermindering van profibrotische markers als TGF-β/Smad en inductie van fibrogenolyse door verbetering van de MMP/TIMP-1 balans. Tevens wordt de anti-oxidant capaciteit van de lever verbeterd door de GSH/GSSG ratio te verhogen. Esculetin is een non-competitieve remmer van 5-lipoxygenase en 12/15-lipoxygenase. Deze enzymen zijn essentieel voor de synthese van leukotriënen en lipoxines, welke sterke pro-inflammatoire signaalmoleculen zijn. Onze resultaten suggereren dat de anti-fibrotische eigenschappen van esculetin, in ieder geval ten dele, lopen via remming van lipoxygenases, bijv. 5-LO en/of 12/15-LO.

In **hoofdstuk 5** presenteren we een vervolgstudie van het anti-fibrotische effect van esculetin, waarin de optimale toedieningsroute en duur van de behandeling met esculetin worden bepaald. Esculetin werd gestart als leverschade en het ontstaan van fibrose al was ingetreden, om de klinische situatie van patiënten met (chronische) leverziekten zoveel mogelijk na te bootsen. Esculetin werd gedurende maximaal één week gegeven; in de tweede, derde of vierde week van behandeling met CCl_4. Opmerkelijk was dat een korte vroege behandeling met esculetin leverschade verminderde (lagere serum ASAT/ALAT waarden) alsmede de progressieve lever fibrose remde. Intraveneuze toediening van esculetin was het meest effectief in verminderen van serum transaminases, stellaatcel activatie markers (Collagen 1a1 en α-SMA expressie), alsook collageendepositie. Blijkbaar heeft esculetin een sterk en langdurig therapeutisch effect in CCl_4-geïnduceerde leverschade en fibrose in muizen.

In **hoofdstuk 6** worden de resultaten van dit proefschrift bediscussieerd in het licht van de huidige stand van zaken in de literatuur wat betreft lever inflammatie en lever fibrose. Tevens wordt hierin een vooruitzicht gegeven voor de toepassing van onze bevindingen in de behandeling van patiënten met leverziekten.

Samenvattend zijn natuurlijke producten, die beschikbaar zijn in miljoenen planten en kruiden, een rijke bron voor ontwikkeling van medicijnen. In dit proefschrift zijn de therapeutische componenten van één van deze medicinale kruiden, *Ipomoea* *stolonifera*, aangetoond. Tevens verhelderen we de mechanismen hoe dit fulminante leverontsteking en lever fibrose onderdrukt. Om deze kennis toe te kunnen passen in klinische trials, zijn aanvullende studies nodig.

Appendix

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A

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Dear Shiva, thank you so much for helping. Although we only have been together for two years, you give me a lot of care and help. I still remembered the first time you are in the lab. Now 4 years passed, time also changed you to be a more open mind and talkative person. I love your attitude of life. Thank you very much for our collaborated work with esculetin. And I wish you my best for your future life and your new career in England!

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致 谢

博士论文已初成，历时四年的博士学习也终告尾声。中国有句古话叫做“十年寒窗”，历经“宝剑锋从磨砺出，梅花香自苦寒来”，在我三十而立之年后，能取得荷兰格罗宁根大学的博士学位，为我的读书生涯画上圆满的句号，我深感欣慰。

2011 年 4 月 22 日，我独自一人来到荷兰，来到格罗宁根这个安静舒适，充满欧洲气息的城市，开始我异国求学之旅。起初，我真的有些担心自己能否在这里生存下来，但荷兰人的友善包容，还有 MDL 大家庭的关心帮助，让我感受到家的温暖。在这里，我非常愉悦地生活学习，并体验了一种全新的生活方式，没有太大的压力，周末可以出海钓鱼，Lab day 可以打高尔夫、划船，假期可以四处游玩，领略各地风情。更有机会到各国去参加国际学术会议。我真的很喜欢这里，当然除了有些时候天气不好。 我最喜欢是进入四月的格罗宁根，鲜花、绿草，还有女王节的橙色（现在是国王节），装点得整个城市非常美。两年的时光，Groningen 给我留下了难忘的印象。由于联合培养博士的项目，2013 年 5 月我又返回中国汕头，完成另一半的实验内容。

致我的敬爱导师及汕头大学医学院

在国内两年时间，我得到导师石刚刚教授的悉心指导和鼎力支持，使我能有开阔的平台去完成自己的设想和实验。在这里，我感谢母校汕头大学医学院，感谢汕医药理教研室，更由衷地感谢石老师对我硕士及博士长达七

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年的无私指导和深厚的师生情谊。石老师，感谢您！祝愿您身体健康！

致帮助我完成课题的每一位

我并不算是一个聪明的人，但每做一件事就想把它做好。我能如期完成这项博士课题还要感谢汕医很多其他的老师，实验员，师兄师姐，师弟师妹，在这里请允许我向所有帮助过我的每一个人诚挚地说一声：“谢谢！” 特别感谢附一体检中心杨佩璇主任的提供测试所有生化学指标；感谢附一中心实验室蔡博治帮助多次组织切片；感谢程訸，姜霁洋，沈岱菲，李丽君在动物实验上大力帮助;感谢周巧玲，罗夏炯，陈伟东在PCR，western blot的帮助；感谢汕医动物实验中心的谢仰民老师在动物实验上提供的方便；感谢药理教研室陈一村，张艳美，高分飞，牛永东老师的指导。感谢汕医黄东阳院长，霍霞处长，张建军科长的关心。由衷祝愿老师们工作顺利，师弟师妹们锦绣前程！

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致我即将出生的女儿

小麦子，好开心你能以这样一种独特的方式参加妈妈的博士答辩，和妈妈共同经历见证这个特别的日子。你的到来，是上帝赐予我和爸爸的礼物，也同时是我们进一步的成长的开始，我们细细地体会孕育你这个小生命的奇妙，也慢慢地学习理解为人父母的不易。这段时间，诸事繁多压力好大，对你也是呵护不够，但是我们的小麦子真的好懂事，在妈妈最难的时候，静静地陪着我一起走过。在这里，我也要感谢你，爱你小麦子！我们期待你的出生！

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Appendix

Biography

Xueting Bai was born on August 19, 1984 in Taiyuan, Shanxi, China. She was born in an inland city, and therefore, from her youth, she always dreamt to go South and see the sea. Her dream became true when she graduated from college and entered Shantou University Medical College for her master in September 2008. Shantou is located on the coastline of the South Sea of China. During her three years of study, she was immersed into the culture of research and science and also the first results (published papers) gave her more confidence to continue her studies.

In 2010, fortunately, there was a fantastic opportunity to join the sandwich PhD program in collaboration with the University of Groningen. She grasped the opportunity with both hands and started a brand new life in Groningen, the Netherlands. In Groningen, she called herself Jackey. Everybody knows her by this name and all like her very much because of the eternal smile on her face. She also enjoyed many amazing experiences, like traveling, dancing and fishing. She likes photography very much and all her emotions and feelings were recorded and presented, although sometime she was alone. In 2013, she had the day of her life: marriage ceremony on July 29. She was so happy and excited to be a bride and get married to her right MAN, Yunsong Yan. This was definitely an unforgettable day in her whole life.

Dedicated to her belief and with hard work, she finally finished all experiments in China and Holland, and was rewarded the doctorate degree from the University of Groningen. During the 4 years of her PhD, she focused her research on the investigation of natural compounds for the treatment of hepatic inflammatory disorders and liver fibrosis. Many studies were performed using mice and it was extremely hard time to raise and care for the mice: "Actually, you have to treat and care for them as your babies every day in order to have good results" she said. These experiences could be very useful in the next step of her career.

In 2015, she moved to Milan, Italy and lived together with her husband. It was really a new start for her as a wife and in November 2016, she will become mother. Her life will again change very much when the little girl comes. Good luck, Jackey! God bless you!